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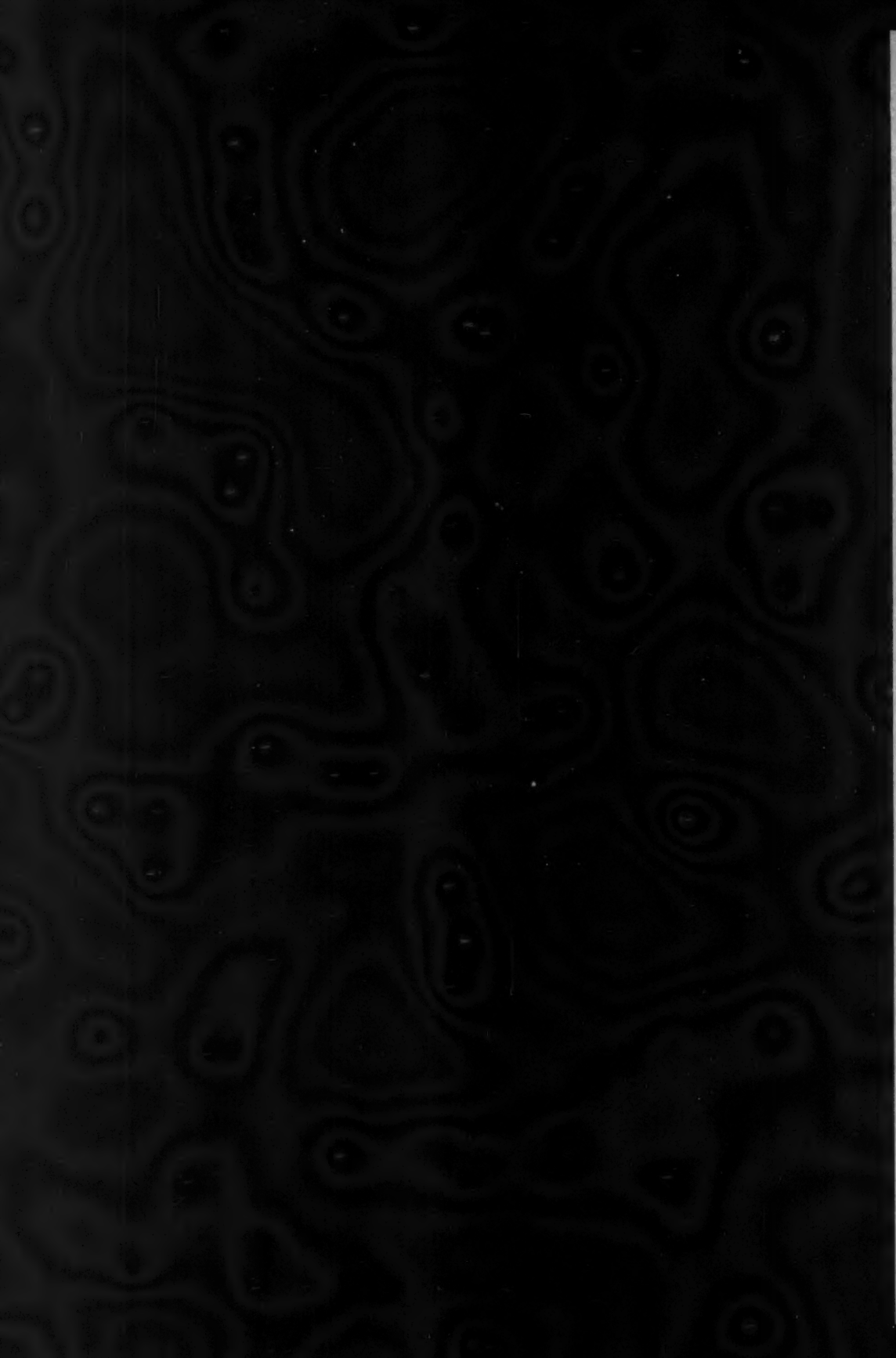

ENDOCRINOLOGY

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EFFECTS OF ACTH ON PREGNANT MONKEYS AND THEIR OFFSPRING

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THIS investigation of the effects of ACTH on the pregnant rhesus monkey and her offspring was an outgrowth of studies by Fleming and Ransohoff[1] on the effects of this hormone on development of erythroblastosis fetalis.[2] When the latter study was initiated (August 1951), there were no published reports on the effects of ACTH on erythroblastic phenomena or on the development of the fetus. Neither was there information on the passage of this hormone from the maternal to the fetal circulation or on the effects of administration of ACTH to the mother on adrenals and other organs of the offspring. In view of the likelihood that for various reasons ACTH would be administered during pregnancy, it seemed desirable to define effects of such treatment on mother and offspring. The availability of a considerable group of pregnant monkeys[3] afforded a unique oppor-

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[1] John G. Fleming, Assistant Professor of Obstetrics, and William Ransohoff, Instructor in Medicine, College of Medicine, University of Cincinnati.

[2] These clinical studies were initiated in August 1951 on the premise that administration of ACTH would improve the consequences of RH sensitization by reducing both the formation of RH antibody in the maternal circulation and the reactivity of the fetal tissues. The first patient to receive protracted treatment with ACTH had a history of five successive pregnancies, each terminating in a non-viable erythroblastic child. This individual was given 20 mg. of ACTH daily from the 25th to the 37th week of her seventh pregnancy. She delivered a full-term viable child with only slight evidence of erythroblastosis. Despite this, the child died approximately 24 hours after delivery. The question was raised as to whether the infant had died from excessive stimulation by ACTH which had passed the placental barrier.

tunity of examining these questions in a primate. This report deals with the results of this investigation.

MATERIALS AND METHODS

Seven pregnant rhesus monkeys were treated with ACTH[4] during the latter parts of their gestation periods, daily doses of 20 mg. being given for intervals ranging from 17 to 67 days. The hormone was administered intramuscularly, 10 mg. at 8 A.M. and 10 mg. at 5 P.M. Another female, mistakenly thought to be pregnant, was treated similarly with ACTH for 133 days. She served as an indicator of the effects of this hormone on a non-pregnant animal. Eight untreated pregnant control monkeys were observed during the last 29 to 120 days of their gestation periods. Two other pregnant controls were observed only at the time of delivery.

All of these monkeys were weighed prior to and once weekly during the treatment or observation periods and again after parturition. Hematological studies, including total erythrocyte, total leucocyte and differential white cell counts, hemoglobin and hematocrit determinations, were carried out at each of these times. As a measure of effectiveness of ACTH administration, eosinophil counts were made before the 8 A.M. treatment, and again 4 to 6 hours later. Similar studies on the babies were made once within 12 to 24 hours after delivery.

All seven of the ACTH-treated females, their four surviving babies, three control mothers and all ten control babies were sacrificed within 24 hours after delivery. The non-pregnant female was sacrificed after 133 days' treatment with ACTH. At autopsy, bone marrow from the distal end of the femur was suspended in monkey serum and smears were prepared. Adrenals, kidneys, spleens, thymus glands (of babies only), thyroids and pituitaries were weighed. Specimens of these and other organs, including bone marrow, were fixed in Zenker-formol. Paraffin sections were made and stained with Lillie's hematoxylin and eosin. In addition, Maximow-stained sections of bone marrow and spleens were prepared. Pituitaries were sectioned serially and stained with Mallory's method, with Weigert's iron hematoxylin and Gomori's trichrome stain (1950) and with Gomori's aldehyde fuchsin (1950) alone or counterstained with the trichrome stain. Sections of pancreas were treated with Gomori's alum hematoxylin phloxine for differentiation of islet cells.

RESULTS

I. *General responses to ACTH treatment*

1. *Effect of ACTH treatment on body weights of mothers and babies.* The untreated pregnant control monkeys gained varying amounts of weight during the latter part of pregnancy, except in two instances where no change or an insignificant change occurred (Table 1). Weight changes in

[3] The monkeys (Macaca mulatta) were members of a colony of about 400 animals maintained at The Christ Hospital Institute of Medical Research, about a hundred of which were sexually mature females. Although females were usually isolated, chance pregnancies had occurred at the rate of 4 to 8 per year, and usually resulted in normal offspring.

[4] Armour's Acthar Gel.

the ACTH-treated pregnant monkeys were more variable, two gaining significantly, one losing, the other four changing little if at all (Table 1). The nonpregnant ACTH-treated monkey gained weight (Table 1). There was no correlation between the weight changes and the duration of the ACTH treatment. Others workers, using non-pregnant laboratory animals, have reported varying effects of ACTH on body weight, from little or no effect to marked loss. Recently Knobil, Hofmann and Greep (1953) reported that 80 mg. daily doses of ACTH, administered to rhesus monkeys for periods of 6 to 42 days, did not affect body weight.

TABLE 1. WEIGHT CHANGES IN UNTREATED AND ACTH-TREATED PREGNANT MONKEYS

Monkey No.	Days of observation	Days of treatment	Pre-treatment body weight, kg.	Pre-parturition body weight, kg.	Weight change during observation period	
					Kg.	Per cent
3587	29	None	7.4	7.7	+0.3	+ 4.0
4065	40	None	7.0	7.1	+0.1	+ 1.4
3548	57	None	7.1	7.1	0	0
4621	67	None	6.2	8.0	+1.8	+29.0
4631	80	None	5.8	6.5	+1.7	+29.3
3610	109	None	5.9	6.3	+0.4	+ 6.8
5108	120	None	5.2	6.3	+1.1	+21.1
5109	119	None	5.3	6.7	+1.4	+26.4
4728*	17	17	5.0	4.7	−0.3	− 6.0
4717	21	21	5.4	5.8	+0.4	+ 7.4
4725	29	29	5.1	5.0	−0.1	− 2.0
4683*	34	34	4.9	4.9	0	0
4641	65	65	4.8	5.4	+0.6	+12.5
4633	67	67	5.8	5.9	+0.1	+ 1.7
4677*	67	67	6.2	6.1	+0.1	− 1.6
4861†	133	133	10.2	11.4	+1.2	+11.8

* Aborted late in pregnancy.
† Non-pregnant.

All ten control mothers delivered healthy babies. The birth weights of eight of these were almost identical, ranging from 0.40 kg. to 0.45 kg. (Table 2). Of the seven ACTH-treated mothers, three delivered dead macerated fetuses late in their term of pregnancy, after 17, 34, and 67 days of treatment. Two of these mothers had moderately advanced tuberculosis; this and/or the frequency with which the animals were handled for purposes of hormone administration may have been responsible for the abortions. However, interruption of pregnancy in rabbits and mice, by treatment with ACTH during the latter half of pregnancy, has been reported by Robson and Sharaf (1952).

The remaining four ACTH-treated mothers gave birth to apparently

TABLE 2. BODY AND ORGAN WEIGHTS OF OFFSPRING FROM UNTREATED AND ACTH-TREATED MONKEYS

Baby No.	Days of ACTH treatment (mothers)	Body weight, kg.	Adrenals		Thymus		Thyroids		Pituitary	
			Combined weight, gm.	Per cent body weight	Weight, gm.	Per cent body weight	Weight, gm.	Per cent body weight	Weight, gm.	Per cent body weight
4718 B	None	0.40	0.421	0.105	0.611	0.152	0.106	0.020	0.015	0.0038
2558 B	None	0.30	0.369	0.123	0.453	0.151	0.117	0.039	0.015	0.0050
3587 B	None	0.45	0.707	0.157	1.041	0.231	0.137	0.030	0.020	0.0045
4065 B	None	0.40	0.386	0.096	0.587	0.146	0.128	0.032	0.014	0.0036
3548 B	None	0.42	0.670	0.159	0.502	0.119	0.089	0.021	0.017	0.0040
4621 B	None	0.40	0.599	0.149	0.305	0.076	0.093	0.026	0.014	0.0034
4631 B	None	0.40	0.322	0.080	1.199	0.299	0.122	0.030	0.014	0.0034
3610 B	None	0.42	0.601	0.143	0.489	0.116	0.095	0.022	0.017	0.0039
5108 B	None	0.50	0.328	0.065	0.856	0.171	0.080	0.015	0.012	0.0024
5109 B	None	0.43	0.209	0.048	1.556	0.361	0.143	0.033	0.017	0.0037
Average		0.41	0.461	0.125	0.760	0.182	0.111	0.026	0.016	0.0035
4717 B	21	0.40	0.538	0.134	0.382	0.095	0.103	0.025	0.013	0.0033
4725 B	29	0.35	0.209	0.063	0.296	0.084	0.124	0.035	0.015	0.0041
4641 B	65	0.37	0.160	0.043	0.240	0.064	0.096	0.020	0.014	0.0038
4633 B	67	0.42	0.080	0.019	0.327	0.077	0.070	0.016	0.018	0.0043
Average		0.38	0.247	0.064	0.311	0.080	0.098	0.028	0.015	0.0038

healthy babies, after 21, 29, 65 and 67 days of treatment. The weights of these offspring ranged from 0.35 kg. to 0.42 kg. Although two were smaller than the majority of controls, the weight comparison was favorable (Table 2).

2. *Hematological studies.* Eosinophil counts in the pregnant controls showed some variations but these were no different than those noted previously in non-pregnant monkeys in this laboratory, or by Ainslie, Francis and Brown (1951), who noted a variation of "approximately 50%" from the grand average. Administration of ACTH resulted in a sharp decrease in eosinophil numbers within 4 to 6 hours, a good indication that sufficient ACTH was being given to induce adrenal stimulation. This depressive effect usually persisted for at least 8 hours after ACTH administration.

Comparison of data from ACTH-treated and untreated pregnant animals showed no real differences in the formed elements of the peripheral blood nor in the values of blood sugar and serum sodium and potassium. Similar findings in part have been reported after ACTH treatment of non-pregnant monkeys. Ainslie, Francis and Brown (1951) found no changes in fasting blood sugar values or glucose tolerance curves. Knobil, Hofmann and Greep (1953) found no significant changes in hematocrit and the various blood constituents.

All pregnant animals, irrespective of treatment, exhibited anemia of pregnancy. There were reductions in numbers of circulating erythrocytes and leucocytes, hemoglobin levels and hematocrits, as compared with those seen repeatedly in non-pregnant monkeys.

Hematological studies done on the babies shortly after delivery indicated

that ACTH given to the mothers had no effect on the immediate post-parturition blood picture of the baby.

3. *Effects of ACTH on bone marrow.* Differential counts in marrow smears showed that ACTH had no effects on the proportions of erythrocytic and neutrophilic elements. Marrow sections of four monkeys treated for 29, 34, 65 and 67 days showed an increased density of hemopoietic elements and a decreased amount of fat as compared with sections of control monkeys or of two treated for 17 and 21 days, and one treated for 65 days (no. 4641). There was some indication, therefore, that ACTH treatment of longer duration caused increased cellularity of bone marrow (although no. 4641 above was an exception). Hudson, Herdan and Yoffey (1953) reported a similar effect on marrow in guinea pigs. However, Baker and Ingle (1938) noted marrow atrophy in rats after large doses of ACTH.

Marrow smears of the two babies whose mothers had been treated with ACTH for 21 and 29 days showed various cell types in percentages comparable to those of control babies. However, the two babies whose mothers were treated for 65 and 67 days showed a 21.0% decrease in erythroid elements and a 22.2% increase in neutrophilic myeloid elements. Marrow sections of these two babies showed a denser aggregation of hemopoetic tissue and less fat.

II. *Histological Studies*

The most striking effects of the administration of ACTH were the alterations in the adrenal glands of both mothers and babies. Some changes were seen in the spleens and lymph nodes of mothers, and in the thymus glands of babies. Changes in other organs were either minimal, questionable, or absent.

Adrenal glands, mothers. There were individual differences in response to ACTH. Three of the seven ACTH-treated mothers had adrenal glands whose absolute and relative weights were greater than those of controls; one was slightly larger (Table 3 and Figs. 1–4). These mothers had been treated for 17, 34, 67, and 67 days. The other three, treated for 21, 29 and 65 days did not show such gross hypertrophy. The adrenals of the non-pregnant monkey, treated for 133 days, showed a pronounced hypertrophy of the same magnitude as found in the pregnant treated animals.

Regardless of whether or not hypertrophy had occurred, all adrenals of ACTH-treated monkeys presented histological evidence of the effects of this hormone. The glomerular zone of the adrenals of the mother treated with ACTH for only 17 days was like that of the controls, with compactly arranged cords of lightly granular cells with vesicular nuclei (Fig. 5). In all other ACTH-treated mothers, the glomerular cells were more variable

TABLE 3. ORGAN WEIGHTS OF UNTREATED AND ACTH-TREATED MONKEYS (POSTPARTUM)

Monkey No.	Days of treatment	Body weight postpartum, kg.	Adrenals		Thyroids		Pituitary	
			Combined weight, gm.	Per cent body weight	Combined weight, gm.	Per cent body weight	Weight, gm.	Per cent body weight
4718	None	4.7	1.270	0.027	0.532	0.011	0.070	0.0014
5108	None	5.2	1.400	0.027	0.570	0.011	0.083	0.0015
5109	None	5.6	0.815	0.015	0.530	0.010	0.087	0.0015
Average		5.1	1.162	0.023	0.544	0.011	0.080	0.0015
4728	17	4.2	2.174	0.052	0.274	0.007	0.064	0.0015
4717	21	5.0	1.331	0.027	0.504	0.010	0.076	0.0015
4725	29	4.5	0.891	0.020	0.581	0.013	0.099	0.0021
4683	34	4.1	2.735	0.067	0.430	0.010	0.066	0.0016
4641	65	4.6	1.160	0.025	0.335	0.007	0.070	0.0015
4633	67	5.5	1.504	0.028	0.462	0.008	0.108	0.0019
4677	67	5.3	2.808	0.053	0.922	0.011	0.068	0.0012
Average		4.7	1.800	0.039	0.501	0.017	0.079	0.0016
4861*	133	11.4	5.740	0.050	0.128	0.006	0.144	0.0012

* Non-pregnant.

in appearance, the zone was irregular in width, uneven along its inner border, and contained numerous irregular spaces, often with debris or desquamating or degenerating cells (Fig. 6).

The majority of cells in the outer fascicular zone of adrenals of pregnant controls were finely vacuolated. The characteristic spongy appearance of this zone was seen only in scattered groups of cells (Fig. 5). The outer fascicular zones of all adrenals of mothers treated with ACTH, and of the non-pregnant control treated with ACTH, were similar in having large cells without any vacuolation (Fig. 6). Cytoplasm was compactly granular, nuclei were more variable in size and in density than in controls. There was no generalized increase in mitotic activity. Cells in the deep fascicular zone were somewhat smaller in size, although still larger than in the controls, and also compactly granular. Sinusoids here were prominent. Thus, adrenal glands of monkeys in advanced pregnancy responded to prolonged ACTH treatment in much the same way as those of a non-pregnant monkey or of other species including man (Baker, 1952; O'Donnell, Fajans and Weinbaum, 1951).

Adrenal glands, babies. The effects of ACTH treatment of the mothers were likewise strikingly demonstrated in the adrenals of the babies but

⟿

EXPLANATION OF PLATE I

Adrenal glands of monkey mothers after ACTH treatment. Lillie's hematoxylin and eosin, ×9.

FIG. 1. 4718, control.

FIG. 2. 4683, ACTH for 34 days.

FIG. 3. 4633, ACTH for 67 days.

FIG. 4. 4677, ACTH for 67 days.

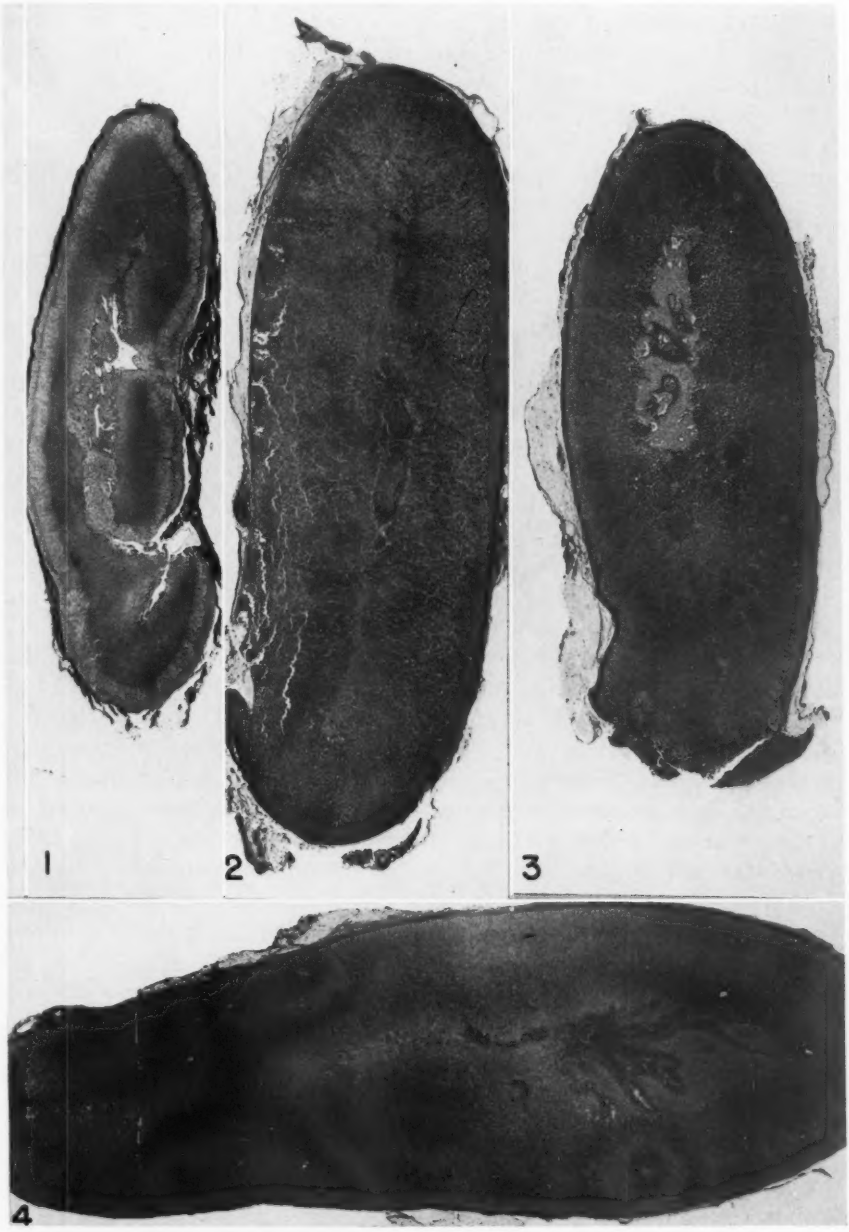

PLATE I

in an entirely different manner. Treatment of the mother for 21 days prior to delivery had no effect on the fetal adrenals, but after treatment for 29, 65 and 67 days, a progressive decrease in size and weight was evident (Figs. 7, 8, 9, 10 and Table 2).

The adrenals of control babies (Figs. 11, 12) differed from adult control glands in that the outer fascicular zone was composed of small compactly granular cells, and the wider deep fascicular zone of much larger cells (Fig. 11), actually huge in some instances (Fig. 12). In these latter cells, the cytoplasm was finely vacuolated, giving this region something of the appearance of a zona spongiosa.

The adrenals of baby no. 4717, whose mother had been treated with ACTH for 21 days, were much like the controls. Those of baby no. 4725, treated for 29 days, were grossly smaller, weighed less (Fig. 9 and Table 2) and the outer fascicular cells were somewhat smaller than those in controls. The adrenals of babies no. 4641 and no. 4633, of mothers treated for 65 and 67 days respectively, were much smaller (Fig. 10) and quite different in structure (Figs. 13, 14). The glomerular zone was wider than in the controls, with large, closely-packed granular cells. This passed into an atrophic outer fascicular zone approximately equal in width to the glomerular zone in baby no. 4641, but more narrow (more atrophic) in baby no. 4633. Cells were small, with dark nuclei that occupied most of the cell volume, and with a narrow rim of densely granular cytoplasm. Cells of the deeper fascicular zone were somewhat larger than those in the outer fascicular zone, but still very much smaller than comparable cells in the controls (compare with Figs. 11 and 12). A reticular zone was not apparent.

These two pairs of adrenal glands, from babies of mothers treated for 65 and 67 days, exhibited structural changes similar to those produced by cortisone in rats and man (Ingle, 1950; Winter, Silber and Stoerk, 1950; and O'Donnell, Fajans and Weinbaum, 1951). This suggests that the ACTH administered to the mother does not pass the placental barrier, but that the excessive cortisone-like substances liberated by the hypertrophied

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⟿

EXPLANATION OF PLATE II

FIG. 5. Adrenal gland, 4718, control (untreated) mother.

FIG. 6. Adrenal gland, 4633, ACTH for 67 days. Hematoxylin and eosin, ×160. Fig. 6 shows spaces in the glomerular zone, irregular glomerular-fascicular boundary and absence of the transitional zone, and hypertrophied, granular cells in the broad outer fascicular zone. a = glomerular zone, b = transition zone, c = outer fascicular zone, d = deeper fascicular zone.

FIGS. 7–10. Adrenal glands of baby monkeys, hematoxylin and eosin, ×9. Fig. 7. 4718 B, control. Fig. 8. 3548 B, control, larger gland than fig. 7. Fig. 9. 4725 B, mother treated with ACTH for 29 days. Fig. 10. 4633 B, mother treated with ACTH for 67 days.

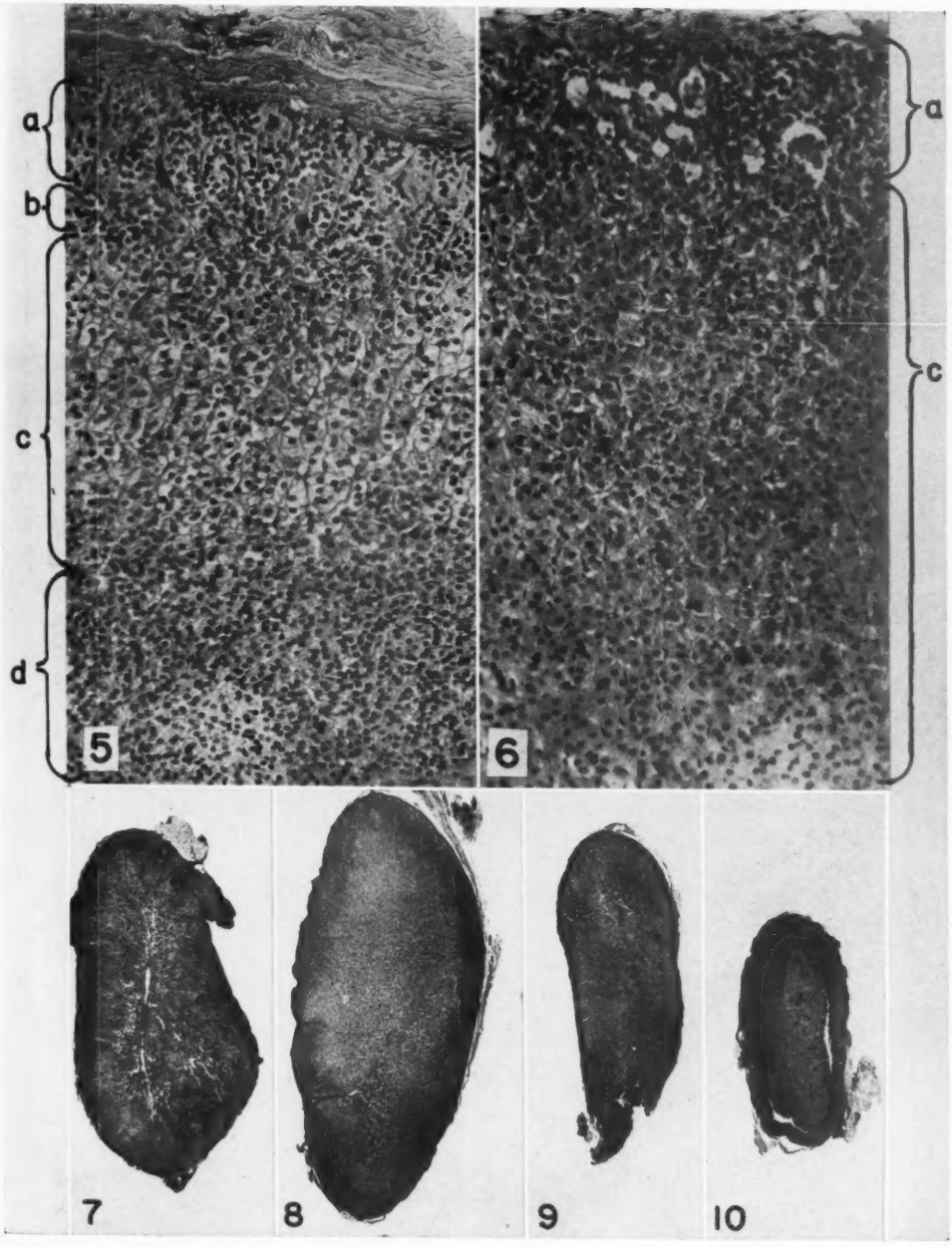

PLATE II

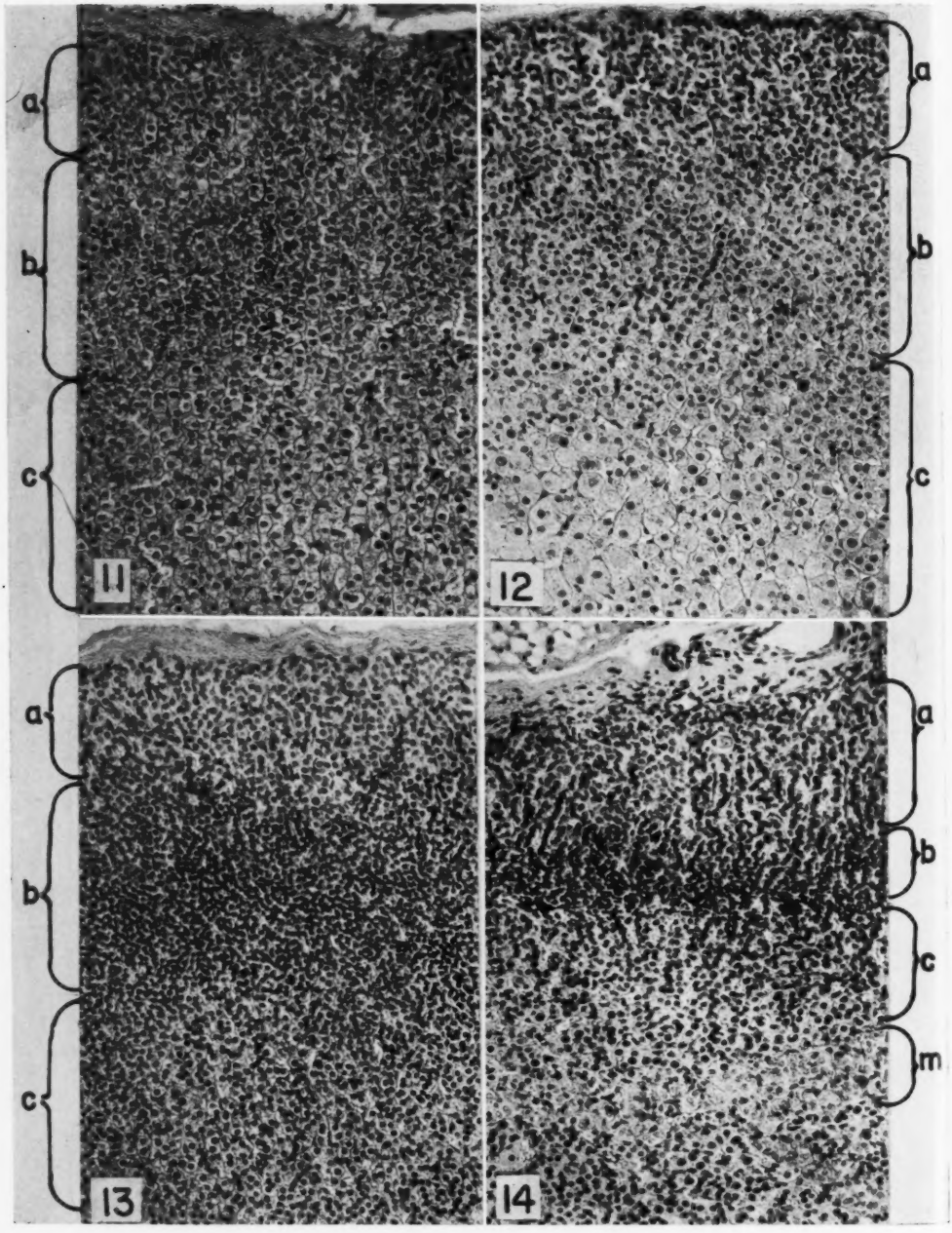

PLATE III

glands of the mother are able to do so, and exert their effect on the fetal adrenal glands.

Thymus glands. Maternal thymus glands appeared well regressed and tissue was not removed at autopsy. Thymus glands of the babies of ACTH-treated mothers were smaller than the control glands (Table 2). The thymus glands of babies no. 4641 and 4633, from mothers treated 65 and 67 days, had many small cellular Hassall's corpuscles as in the controls, but in addition, showed larger Hassall's corpuscles with a greater degree of cornification and degeneration, and a marked reduction and atrophy of cortical lymphoid tissue, accompanied in places by an invasion of numerous neutrophils. Thus thymus glands of these babies also showed a cortisone effect, for thymus atrophy as the result of administration of cortisone had been noted in other animals (see Ingle 1950). Although it has been demonstrated that ACTH also causes thymic atrophy (see review by Dougherty 1952), this is apparently an indirect effect, brought about through stimulation of the adrenal cortex (Bruce, Parkes and Perry, 1952).

Spleens and lymph nodes. Spleens of pregnant animals, treated with ACTH for the longest periods of time (65 or 67 days), showed suppression of activity, as has been observed repeatedly in other non-pregnant laboratory animals (see Ingle 1950) and very markedly in our non-pregnant monkey. Splenic corpuscles were of moderate, size, without germinal centers, or with minute centers showing a preponderance of large lymphocytes. Lymph nodes showed a similar suppression of activity. Neither spleens nor lymph nodes of babies of ACTH-treated mothers showed any striking differences from those of the controls.

Pituitary glands, mothers. Weights of pituitaries from ACTH-treated mothers were within the range of controls except for two which varied somewhat more (Table 3 last column). There was thus no decrease in size of the pituitary such as observed in non-pregnant rats after prolonged ACTH treatment (Baker, 1952). However, the pituitary of our non-preg-

←

EXPLANATION OF PLATE III

Adrenal glands of baby monkeys from control (untreated) and ACTH-treated mothers. Hematoxylin and eosin, ×160. a = glomerular zone, b = outer fascicular zone, c = deeper fascicular zone, m = medulla.

FIG. 11. 4718 B, control. Outer fascicular zone has granular cells, deeper fascicular zone has larger vacuolated cells.

FIG. 12. 3548 B, control. Same but cells of deeper fascicular zone are still larger and more vacuolated.

FIG. 13. 4641 B, mother treated with ACTH for 65 days.

FIG. 14. 4633 B, mother treated with ACTH for 67 days. In both, glomerular zone is wider, outer fascicular zone has atrophic cells, deeper fascicular zone has small granular cells.

nant monkey, although grossly larger than the others, showed a decrease in size in proportion to body weight after more prolonged ACTH treatment (Table 3, no. 4861).

There were no constant histological differences between the pituitaries of control and ACTH-treated monkeys, thus supplementing similar conclusions reached by others working with non-pregnant rats (Baker, 1952; Koneff, 1944; Winter, Silber and Stoerk, 1950). The only specific change noted was a reduction in the number of beta cells (thyrotropic basophils) in the pituitaries of two of the longer-treated pregnant monkeys (nos. 4683 and 4633). Such a decrease was not found, however, in the other two long-treated pregnant monkeys (nos. 4641 and 4677) nor in the non-pregnant one treated for 133 days. Pronounced qualitative changes in structure of basophils, as described for older rats by Koneff (1944), Golden and Bondy (1952) and in the human pituitary by Golden, Bondy, and Sheldon (1950) and Lacqueur (1950) were not seen.

Pituitary glands, babies. Weights of the four baby pituitaries from ACTH-treated mothers were similar to each other and to the pituitaries of control babies (Table 2). No significant or constant histological alterations were noted. The pituitary of baby no. 4633 (mother treated for 67 days) showed a reduction in the number of beta cells but the comparable baby (no. 4641) did not show such a decrease.

Pancreas and other organs. Pancreatic acini of ACTH-treated mothers were unaffected by ACTH. Some of the islets of Langerhans of mothers no. 4633 and no. 4677 (treated 67 days) showed enlargement of beta cells and continued proliferation of islet tissue, changes similar to those reported by Baker (1952) in rats. Prolonged ACTH treatment of pregnant monkeys had no significant effects on thyroids, ovaries and Fallopian tubes, livers, kidneys, lungs and heart muscle.

In the babies, presumably subjected to cortisone effects, these same organs showed no outstanding differences (see Table 2 for thyroid weights), a conclusion reached by others working with rats. Winter, Silber and Stoerk (1950) reported normal-appearing thyroids after prolonged cortisone-acetate treatment, although Halmi and Barker (1952) found smaller follicles and taller epithelium. Cortisone had little or no effect on male genital organs (Antopol, 1950; Ingle, 1950; Winter, Silber and Stoerk, 1950).

DISCUSSION

The administration of ACTH to monkeys during the latter part of pregnancy provided several findings of interest. It was apparent that the adrenal glands of pregnant monkeys were able to respond to ACTH in the same way as adrenal glands of non-pregnant animals. Hypertrophy of

adrenal fascicular cells and loss of vacuolation in their cytoplasm was a striking histological effect, as was suppression of activity in the spleen and lymph nodes. These findings in pregnant monkeys are generally in agreement with those reported in other non-pregnant laboratory animals when ACTH has been given over long periods of time.

As indicated previously, the adrenals of the babies of ACTH-treated mothers exhibited striking changes, comparable to those produced by cortisone in adult animals. This finding has been interpreted as a reflection of the increased production of cortisone-like substances by the maternal adrenal and the passage of these materials across the placental barrier, with resultant depression of the activity of the fetal adrenal.

The work of Jones, Lloyd and Wyatt (1951) on cholesterol content of maternal and fetal adrenals of rats offers additional evidence to support our findings. Stimulation of the mothers' adrenals by ACTH caused a decrease of 46% in maternal adrenal cholesterol, but an increase of 55% in cholesterol content of fetal adrenals. This we interpreted to indicate that the increased cortisone from the mother's hypertrophied adrenals, circulating in the maternal blood, passed to the fetus and suppressed the formation of cortisone in fetal adrenals, with consequent accumulation of the cholesterol precursor. In a later more complete report (1953) these authors reached a similar conclusion, that ACTH of moderate doses does not cross the placental barrier of the rat but steroids of maternal origin do pass the placenta and inhibit the fetal adrenal. However, they state that high levels of ACTH in the maternal rat can enter the fetus and produce a stimulation of the fetal adrenal.

SUMMARY

Seven pregnant rhesus monkeys were treated with 20 mg. daily doses of ACTH during the last 17 to 67 days of their gestation periods, one non-pregnant female was similarly treated for 133 days, and ten untreated pregnant monkeys served as controls. This treatment produced a marked reduction in circulating eosinophils. There were no effects on the numbers or distribution of other formed elements, or on the concentrations of sodium and potassium in serum, nor the levels of glucose in whole blood.

Three of the seven pregnant treated monkeys aborted. The other four pursued an uneventful course and gave birth to apparently healthy offspring. All were sacrificed within 24 hours after parturition.

Maternal spleens and lymph nodes showed characteristic suppression of activity in the nodules. These same organs in the babies were unaffected but thymus glands showed cortical atrophy. There were no significant changes in maternal or fetal pituitaries, pancreas, bone marrow, thyroids,

ovaries and Fallopian tubes, livers, kidneys, lungs and heart muscle, nor in fetal testes and epididymides.

Maternal adrenals responded typically to ACTH by hypertrophy of fascicular cells and loss of vacuolation in their cytoplasm. Fetal adrenals, on the contrary, were very small, with enlarged glomerular zones and atrophic fascicular zones—changes similar to those produced in adult adrenals by cortisone. This suggested that excessive amounts of cortical steroids from the enlarged maternal adrenals passed into the fetal circulation and inhibited activity of fetal adrenals.

ADDENDUM

Recently this work was supplemented with five additional monkeys, primarily for the purpose of obtaining lipid stains on the adrenal glands. One pregnant animal served as an untreated control. Three pregnant animals were treated with ACTH, as described previously, during the last 31, 59 and 63 days of their respective gestation periods. All gave birth to living, apparently healthy young. Thus, out of our total number of ten ACTH-treated pregnant monkeys, seven delivered and three aborted. A fifth animal, mistakenly thought to be pregnant, was treated with ACTH for 66 days. Its body weight (6.5 kg.) and length of treatment were comparable to those of the other four animals. All data obtained on these animals agreed closely with those of the original group. Average figures given in Tables 1, 2, and 3 would remain essentially the same by the addition of the new data.

All adrenal glands were fixed in formalin and embedded in gelatin. Frozen sections, 15 microns in thickness, were stained with sudan III, sudan black B and oil red O. The adrenals of the untreated pregnant control showed a concentration of lipid material in the outer one-sixth of the fascicular zone and a scattering of fine granules in the rest of the fascicular zone and in the glomerular zone (Fig. 15). After treatment with ACTH for 31 days, the entire enlarged fascicular zone was filled with lipids, with a somewhat greater concentration in the outer half. A similar concentration was seen in the deeper half of the glomerular zone (Fig. 16). Here, and in all other ACTH-treated mothers and babies, the reticular zone as such was not apparent but cells in this area were likewise filled with lipids. After treatment with ACTH for 59 and 63 days, lipids were still generally dense throughout the enlarged fascicular zone, but small areas of lesser concentration were seen in the outer region and elsewhere. This suggests a beginning of lipid exhaustion. A marked difference was the presence of heavy lipid deposits in the glomerular zone and the adjacent fascicular region (Fig. 17). The lipid content of the adrenals of the non-pregnant animal treated for 66 days was generally similar to that seen in the

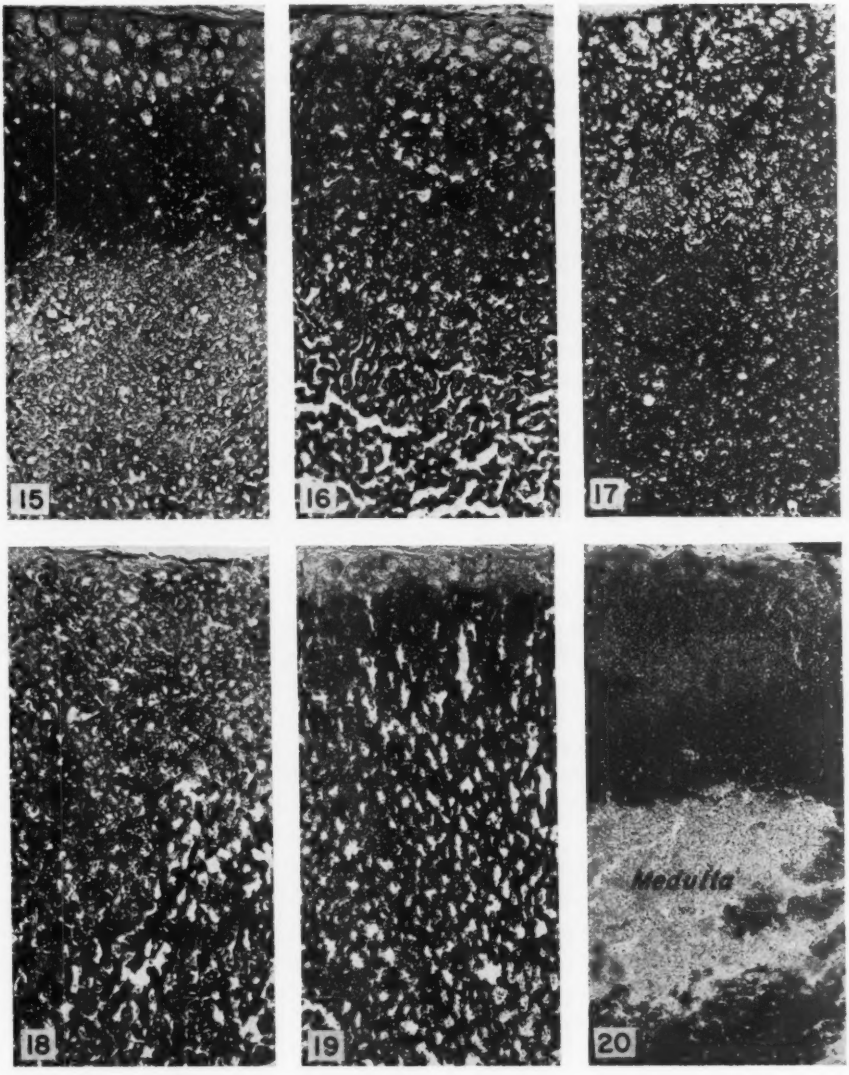

PLATE IV

Adrenal glands. oil red O, ×60.

FIG. 15. 5134, untreated mother.

FIG. 16. 5235, mother treated with ACTH for 31 days.

FIG. 17. 3653, mother treated with ACTH for 59 days.

FIG. 18. 5134 B, baby of untreated mother.

FIG. 19. 5235 B, baby of mother treated for 31 days.

FIG. 20. 3653 B, baby of mother treated for 59 days.

31-day treated pregnant animal. The stimulation of lipid formation by ACTH has been well demonstrated therefore in both pregnant and non-pregant monkeys. Lipid was present in cells which appeared compactly granular with routine stains.

The combined adrenals of the control baby weighed 0.689 gm. The cortex showed fine lipid granules irregularly distributed in the smaller-celled outer fascicular zone, and heavy lipid deposits in the large cells of the wide deeper zone (Fig. 18). After ACTH treatment of the mother for 31 days, the baby's adrenals were reduced in size (combined weights 0.333 gm.) and fascicular cells were markedly smaller. Lipid was present throughout the fascicular zone, being more concentrated in the deeper portion in some areas (Fig. 19). After 59 days of ACTH treatment, both the cortex of the baby's adrenals and the fascicular cells were greatly reduced in size (combined adrenal weights 0.019 gm.). Lipids were still present, however (Fig. 20). The heaviest deposits were seen in the deep fascicular area, and fine granules were present in the outer fascicular zone even though these cells were very small with only a narrow rim of cytoplasm. An intermediate concentration of fine granules was also noted in the glomerular zone where virtually none had been before. The adrenals of the baby after 63 days' treatment of the mother with ACTH were less drastically reduced in size (combined weights 0.181 gm.) but lipid distribution was similar except for a lesser amount in the glomerulosa.

It has been reported by others (O'Donnell, Fajans and Weinbaum, 1951; Stebbins, 1950; Winter, Silber and Stoerk, 1950) that administration of cortisone to both man and animals produced various degrees of lipid diminution in the cortex depending on the dosage employed and the duration of treatment. In our monkeys, the amount of maternal adrenal steroids to which the babies were subjected was apparently not sufficient to deplete the supply of lipids. It is evident, however, that there has been a redistribution. The progressively smaller size of the adrenals and continued shrinkage of cortical cells may give an erroneous impression of the actual amount of lipid in the gland. The apparent density may represent a concentration into a much smaller area. Or the lipid may represent an accumulation of cortical steroid precursors.

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THE INFLUENCE OF ESTROGEN AND PROGESTERONE ON THE ISOMETRIC TENSION OF THE UTERUS IN THE INTACT RABBIT

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A SYSTEMATIC analysis of the properties of uterine muscle at the molecular level was first made by Csapo (1948, 1949, 1950 a and b) and Csapo, Erdös, Naeselund and Snellman (1950) and Csapo and Gergely (1950), and it was found that the final contractile system involving contractile proteins, high energy phosphates and ions corresponds very closely to that occurring in skeletal muscle (Szent-Györgyi, 1945 and 1951). Experiments relating to the effects of ovarian hormones on the uterine contractile system showed that estrogen is responsible for the synthesis of the contractile proteins (Csapo 1950 b), and for the increase in concentration of high energy phosphate compounds which serve as an immediate source of energy (Menkes and Csapo 1952). An effect of progesterone on these muscle constituents could not be demonstrated conclusively, and it appears, therefore, that the influence is manifest at a higher level of organization (Csapo and Corner 1952).

To determine the influence of the ovarian hormones, excised uterine muscle was studied in vitro by isometric techniques and characteristic differences between estrogen- and progesterone-dominated uteri were observed (Csapo and Corner 1952). In any consideration of the working capacity of muscle and of the contractile units involved in motility, it was clearly essential to make isometric as opposed to isotonic recordings. An isotonic system may record maximum activity when a few muscle cells are contracting, but an isometric system records a maximum effect only when all the contractile units are involved. In addition an isometric system distinguishes between the contractile capacities of the individual units under different hormonal and enzymic conditions. The properties of the uterus were investigated by direct electrical stimulation of the muscle. Broadly speaking, when spontaneous muscle activity is studied, two systems are involved, the spontaneous stimulatory system, which may or may not be nervous, and the ability of the muscle itself to respond to these impulses. Results are therefore difficult to interpret since either of the systems may

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be responsible for an altered effect. Hence in these experiments the spontaneous stimulatory mechanism was excluded by substituting an electrical stimulatory system which could be controlled and varied at will.

Parallel experiments have now been carried out in the intact animal to see how close a comparison can be drawn between the isometric properties of uterine muscle manifest *in vitro* and *in vivo*, and to investigate how these properties are modified by the regulating influence of the intact animal.

METHODS

Adult white New Zealand rabbits weighing about 3.5 kg. were castrated bilaterally under Nembutal anesthesia and were treated daily for a week with 25 μ estrogen injected intramuscularly in oil. Experience has shown that the uteri of castrate animals thus treated are capable of developing tension equal to that of uteri taken from natural estrus animals. Thereafter they were divided into two groups and were injected for four days either with 10 μ estrogen for an estrogen-dominated uterus, or with 10 μ estrogen and 2 mg. progesterone for a progesterone-dominated uterus. It was necessary to give estrogen simultaneously with progesterone to preserve the contractile capacity of the uterine muscle. Experiments were carried out the day of the last injection and animals were anesthetized with Dial. A few rabbits were untreated and used three weeks after castration. In the *in vitro* studies already published (Csapo and Corner 1952, 1953) slightly different preparatory hormone dosage was used, but in the series of experiments reported here, one horn of the uterus was excised from each rabbit, and *in vitro* observations, which were similar to those already published, were made by Csapo. His further observations are in the press.

The plexiglas chamber (Fig. 1) which was used in these experiments was modified from the cannula designed by Bell and Robson for their investigation of the spontaneous isotonic motility of the uterus. The chamber was inserted through an incision in the abdomen, and when the uterus had been fixed in contact with the electrodes the abdominal wall was sewn up round the chamber above the flange. The uterus (U) lay inside in the position shown in Figure 1. One pole of the stimulator was represented by the two electrodes labelled B. The screws, which could be connected to the stimulator, were continuous with the rigid platinum wires at the bottom which projected into the central space. Thread passing through small holes in the plexiglas wall on either side of the platinum wires held the uterus pegged onto the two electrodes which thus pierced the uterine tissue and fixed the two ends of the uterine segment. The other pole of the stimulator was connected to electrode A which was a platinum hook inserted down the chimney of the chamber and passing round the uterus midway between the electrodes B. Thus the stimulating current passed between A and the two electrodes B.

In the resting state the uterus lay in the position shown, and when it contracted it flattened and pulled down the central hook A. This hook was attached by thread to the short arm of an isometric tension recorder in which the lever was suspended on a flat torsion spring of steel. The ratio of the long to the short arm of the lever was 37 to 1 and the greatest excursion of the lever, representing maximum tension, corresponded to an actual shortening of the uterine segment of less than 2% of its resting length.

In long term experiments the spontaneous activity and response to electrical stimulation continued unchanged for at least 30 hours. All the experiments reported here, however, were carried out in the first five hours. The voltage applied ranged from $\frac{1}{2}$ volt up

to 20 volts, but voltages higher than 12 were seldom used since maximum tension was developed before this. The duration of the stimulus was in every case 5 seconds. In all the experiments 60 cycle A.C. was used. Records were made on a smoked drum.

RESULTS

The threshold stimulus for an estrogen-dominated uterus was found to be $\frac{1}{2}$ or 1 volt, and for a progesterone-dominated uterus, 1, 2, or sometimes 4 volts. The response to increasing voltage was observed in the two types of uteri by stimulating once at each voltage with an ascending strength starting at $\frac{1}{2}$, 1, 2 and thence in two volt steps until the uterus developed maximum tension (Fig. 2). In the estrogen-dominated uterus the rise in

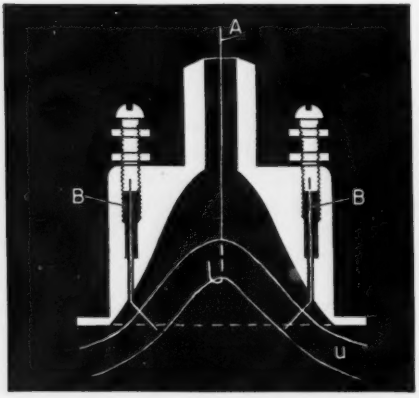

FIG. 1. Cross section of the plexiglas chamber which was inserted in the abdomen to fix the uterus and apply the electrodes. A = Platinum hook representing one pole of the stimulator and attached by thread to the isometric tension recorder. B = Platinum electrodes representing the other pole of the stimulator. U = Uterus.

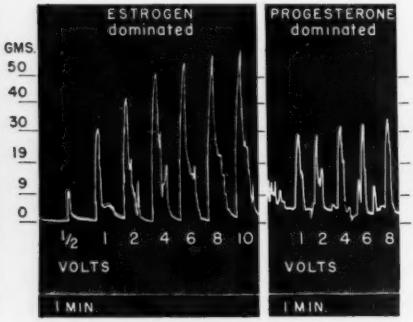

FIG. 2. Tracing showing ascending voltage curves for an estrogen- and a progesterone-dominated uterus. Tension calibrated in grams.

tension was gradual and the maximum was obtained sometimes at as high a voltage as 10. In the progesterone-dominated uterus, however, the rise in tension was abrupt and the maximum tension developed early in the voltage rise. A descending voltage curve was obtained by reducing the voltage in reverse order, and a similar difference between the two types of uteri was observed (Fig. 3). These voltage curve phenomena occur at both 1 and 2 minute frequencies of stimulation.

After a period of rest of at least ten minutes, an initial uniform voltage produced staircases with opposite slopes for the two types of uteri (Fig. 4). With the estrogen-dominated uterus the maximum tension developed

slowly and was obtained after three or four stimuli; but the progesterone-dominated uterus developed maximum tension with the first stimulus and thereafter the tension declined somewhat. The staircase phenomenon, however, can be investigated with greater accuracy by changing the frequency of stimulation (Fig. 5). The estrogen-dominated uterus showed a positive staircase, that is, the higher the frequency of stimulation, the higher the tension developed. The progesterone-dominated uterus, however, developed higher tension with decreasing frequency that is, it exhibited a negative staircase. The castrate uteri developed only a few grams of tension when stimulated and soon lost this power. Hence it was not possible to investigate the staircase phenomenon as was done in the *in vitro* experiments (Csapo and Corner 1952) in which the absence of a staircase was demonstrated.

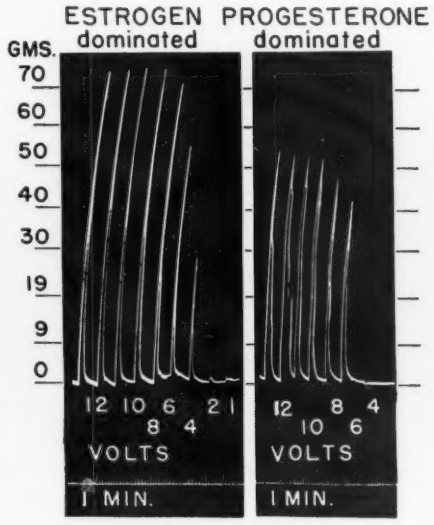

FIG. 3. Tracing showing descending voltage curves for an estrogen- and a progesterone-dominated uterus. Tension calibrated in grams.

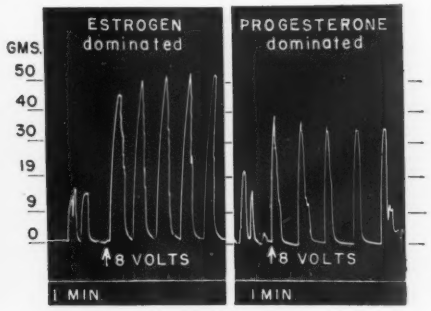

FIG. 4. The staircase phenomenon as shown by the application of electrical stimuli at uniform frequency after a period of rest. Tension calibrated in grams.

In the experiments illustrated by Figure 6, a voltage sufficient to produce maximum tension was applied and the voltage was then halved once between two consecutive stimuli. In the estrogen-dominated uterus this led to a gradual diminution in tension, whereas with the progesterone-dominated uterus the tension fell abruptly and thereafter recovered slightly to maintain a somewhat higher tension.

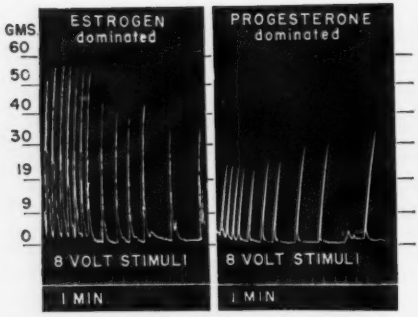

FIG. 5. The staircase phenomenon as shown by altering the frequency of stimulation. Tension calibrated in grams.

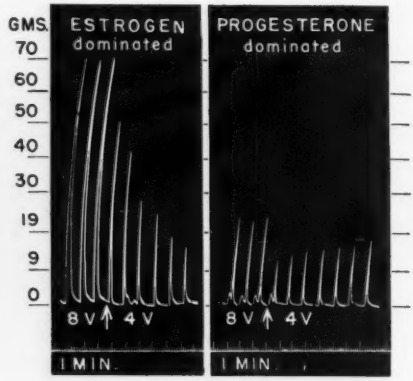

FIG. 6. Tracing showing the effects of halving the stimulating voltage on an estrogen- and a progesterone-dominated uterus. Tension calibrated in grams.

DISCUSSION

The properies of uterine muscle described here correspond in character to those observed *in vitro* (Csapo and Corner 1952, and Csapo, to be published). In the *in vivo* preparation the muscle properties are somewhat modified by the regulating influence of the intact animal as compared with the *in vitro* preparation in which the properties of the muscle per se are studied, and hence the tracings obtained *in vivo* show less marked and less regular effects. The character of the muscle behavior seen during electrical stimulation changes markedly according to whether the hormone dominating the uterus is estrogen or progesterone. This change has been related experimentally to an alteration in the intracellular cation balance (Corner and Csapo 1953, and Horvath, in press) rather than to any direct influence on the muscle proteins responsible for contractility.

In addition to qualitative considerations it is not possible with the present technique to contrast the quantitative aspects of tht two types of uteri since the length of uterus between the two electrodes is not necessarily the same, and since the resting positions of the segments are not identical. In most cases the progesterone-dominated uteri develop apparently less tension than the estrogen-dominated uteri, but since the former are longer and more twisted in situ, this may not be a real difference. Further modifications of the technique have now been adopted, and it is hoped to be able to make some quantitative comparisons at a later date.

SUMMARY

1. Experiments were carried out on the intact rabbit to demonstrate the influence of estrogen and progesterone on the isometric tension developed by the uterus.

2. The pattern of contractility elicited by altering the strength and frequency of electrical stimulation was found to vary considerably according to whether the dominating hormone was estrogen or progesterone.

3. These differences correspond to those already found by Csapo in the isolated preparation.

4. Differences between the two types of uteri are discussed.

Acknowledgments

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APPARENT EXOPHTHALMOS IN THE RAT FOLLOWING CORTISONE TREATMENT OR THYROIDECTOMY

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CLINICAL exophthalmos in man is associated with an increase in the mass of retrobulbar tissues (Dobyns, 1950). Although the etiology of the changes in these tissues is not well understood, it appears that most of the alterations are in the connective tissue, fat, and muscular components. Experimental exophthalmos produced by treatment with pituitary preparations in adult guinea pigs is also associated with an increase in the mass of retrobulbar tissues and with histological changes similar to those seen in human exophthalmos (Smelser, 1937).

Recently exophthalmos has been reported to occur in rats receiving cortisone (Aterman and Greenberg, 1953). Others have reported exophthalmos in rats which had been thyroidectomized (Leblond and Eartley, 1952) or had been fed either iodine deficient (Thompson, 1933) or low salt diets (Yudkin, Farquhar and Wakerman, 1933). Apparently these observations were based only on visual examination of living animals. In all of these studies young animals were used. If one examines these data it is apparent that exophthalmos was observed only in those animals in which growth was retarded. It has been shown that the eyeball and brain continue to grow in young rats deprived of either the thyroid (Scow and Simpson, 1945) or pituitary gland (Walker, Simpson, Asling and Evans, 1950), even though body growth is markedly retarded. Since the rate of eyeball growth is nearly normal in these dwarfs, the ratio of eyeball to body weight may become 3 to 4 times as great as that in normal controls of the same age. It is thus conceivable that the eyeballs would appear more prominent in these dwarfs than in normal rats, not as the result of an increase in the mass of retro-bulbar tissues, but as the result of the marked disproportion between the eyeballs and body size. The present experiments were planned to investigate this possibility in rats dwarfed by cortisone administration and by thyroidectomy. The effect of these treatments on the size of the skull, the orbit, and the orbital tissues was studied.

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MATERIALS AND METHODS

Male Sprague-Dawley rats, individually caged, were fed a modification of McCollum's lactating diet (Boas and Peterman, 1953). They were permitted to drink water and eat *ad libitum*. Five groups of weanling rats were used to study the effect of cortisone treatment and thyroidectomy on the production of experimental exophthalmos. At 28 days of age, the operative procedures were performed and the cortisone therapy begun. Group I consisted of normal rats; Group II, sham-operated controls; Group III, intact rats treated with cortisone; Group IV, completely thyroidectomized rats; and Group V, incompletely thyroidectomized rats. Animals in Group I were autopsied at 28 days of age and those in the remaining groups at 68 days of age.

The animals injected with cortisone were weighed daily. Each received 2.5 mg. of cortisone acetate subcutaneously, as needed, to effectively retard growth. To obtain this effect, they were injected approximately once every two days. The total dosage given each rat averaged 48.6 ± 2.8 mg. (S.E.).

Thyro-parathyroidectomy and sham-thyroidectomy were performed under ether anesthesia. At the termination of the experiment, completeness of thyroidectomy was ascertained by the radioautographic technique of Reinhardt (1942). In the present study, if the mean area of the images produced by the cervical and mediastinal tissue on the two roentgen films, one placed on either side of the tissue, was less than 14 mm.^{2}, the rat was considered to be completely thyroidectomized, even though there was a trace of thyroid tissue remaining in some of the rats. If the image area was larger than 14 mm.^{2}, the rat was considered to be incompletely thyroidectomized.

All rats were killed by exsanguination, under light ether anesthesia, by cutting a carotid artery. After carefully removing the eyelids flush with the orbital rims, and the skin from the top of the head, the orbital contents were removed, separated and weighed in pairs. The eyeballs were removed first by separating them from the conjunctiva, extraocular muscles and optic nerves. The Harderian glands and orbital connective tissue were then removed by incising the conjunctiva along the bony and muscular rim of the orbits and carefully separating them from the orbital walls. The Harderian glands were dissected clean of adherent tissues. The remaining portion, which is referred to as orbital connective tissue, consisted of the extraocular muscles, conjunctiva, the optic nerve and the loose connective tissue elements. The infraorbital glands, which lie on the muscular rim of the orbit, were left in situ.

The volume of the empty orbits, as formed by the skull and temporal muscles, was measured by pipetting a detergent solution (Alconox) into them until the fluid was level with the orbital rim. Before the measurement was made the lower jaw was centered and held shut with an elastic band and the head was tipped so that the orbital rim was horizontal.

The head was cut off at the foramen magnum and the remaining skin, ears, external orbital glands and salivary glands were removed to give a uniform head preparation to weigh. The brain, mandible, tongue, and masticatory muscles were included in the head weight.

A number of rats were set aside at the end of the experiment from representative experimental groups and the specific gravity of the orbital contents *en masse* was determined by a water displacement technique. The mean specific gravity of the orbital contents in all groups (14 determinations) was 1.10 with a range of 1.08 to 1.14, there being no significant differences between groups. An estimate of the mass of tissue which could be contained in the orbit was made by multiplying the orbital volume by the specific gravity of these tissues.

Skull measurements were made with calipers on cleaned, dried, skulls (Figure 3). The soft tissue was easily removed after autoclaving at 15 pounds per square inch for 15 minutes.

RESULTS

Growth of Orbital Tissues: Normal growth curves for the eyeballs, Harderian glands, and orbital connective tissue were obtained by determining weight of these tissues in normal rats which were autopsied at various ages (Figure 1). The weight of each of these tissues increased at a progressively slower rate than did the body weight. This relative decrease in rate of

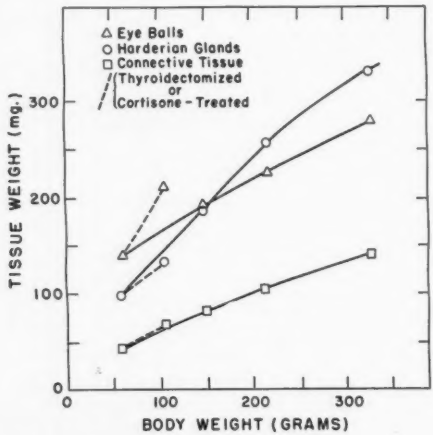

FIG. 1. Relationship of the weight of the Harderian glands, eyeballs, and orbital connective tissue to the body weight. The values for cortisone-treated and completely thyroidectomized groups, being almost identical, are represented by a single point on each curve. The solid lines represent tissue weights of normal male rats; the broken line, cortisone-treated and thyroidectomized rats. Each point on the curves represents 6–8 rats; the standard error for each mean, regardless of the tissue or animal group, was less than ± 13 mg.

growth was most pronounced in the eyeball. The eyeballs/body weight ratio was 0.24% in 56 gm. and 0.09% in 326 gm. normal rats.

Cortisone and Thyroidectomy: This experiment was designed to study the effects of cortisone treatment and thyroidectomy on the development of exophthalmos in the rat. The results are given in Tables 1 and 2.

The final body weight of both the cortisone-treated and the thyroidectomized rats[1] averaged approximately one-half of that of the sham-

[1] Unless indicated otherwise, rats referred to as thyroidectomized are those considered to be completely thyroidectomized (group IV).

operated controls, whereas the head weight in these groups was about three-fourths of that of the control group. This resulted in a larger head/body weight ratio in the dwarfed animals. The eyes of the cortisone-treated and thyroidectomized rats appeared more prominent than those in the sham-operated controls. This apparent eyeball prominence was variable, however, and depended on the width of the palpebral fissure, which could be easily affected by the state of excitement of the animal. The photograph of a thyroidectomized rat in Figure 2 shows the appearance of these dwarfs, when the eyes are wide open.

The total weight of the orbital contents (eyeballs, Harderian glands, and connective tissue) was less in the cortisone-treated and thyroidectomized rats than in the sham-operated controls (Table 1). It can be seen that this

FIG. 2. This photograph of a thyroidectomized rat, about 6 months postoperative, demonstrates the marked prominence of the eyeballs which can be observed at times.

difference was due primarily to smaller Harderian glands and less connective tissue since the eyeball weights in all groups were nearly the same. Since the head/body weight ratio was larger in the dwarfed animals, and their eyeballs were the same size as the controls, the ratio of eyeballs to head weight was greater in the dwarfed than in normal rats of the same age. If one compares the weights of the orbital tissues in the treated or operated rats with those in the normal rats of the same body weight, as is done in Figure 1, it is evident that the cortisone-treated and thyroid-

TABLE 1. EFFECT OF CORTISONE TREATMENT AND THYROIDECTOMY ON THE WEIGHT OF THE HEAD AND OF THE ORBITAL TISSUES[a]

Group	Age (days)	No. of rats	Body weight (gm.)		Head weight		Harderian glands (mg.)	Connective tissue (mg.)	Eyeballs (mg.)	Total orbital contents (mg.)
			Initial	Final	Gm.	% of body weight				
I. Normal	28	6	—	59±3	6.1±.1	10.3±.3	97± 5	43±3	139±2	280± 8
II. Sham-operated	68	8	54±2	211±7	12.9±.3	6.1±.1	260±12	105±4	226±4	591±13
III. Cortisone-treated	68	7	55±2	95±7	8.5±.3	9.1±.4	138± 6	68±3	202±3	407±11
IV. Thyroidectomized (complete)	68	11	52±1	105±5	9.1±.2	8.7±.2	134± 5	66±2	210±2	410± 7
V. Thyroidectomized (incomplete)	68	5	54±3	142±9	10.8±.3	7.8±.4	184± 6	87±5	218±4	489±10

[a] Mean ± Standard Error.

ectomized rats have proportionally larger eyeballs, smaller Harderian glands, and a normal amount of connective tissue.

The orbital volume was smaller in the cortisone-treated and thyroidectomized rats than in normal rats of similar age (Table 2). The difference between the mass of the orbital tissues and the mass that could be contained in the orbit is an estimate of the amount of tissue that protruded, which consisted mostly of eyeball. From this the percentage of the eyeball extending beyond the orbital rim can be calculated. This value was 85% in the 68 day old sham-operated, 77% in cortisone-treated, and 79% in thyroidectomized rats. This would then indicate that there was no exophthalmos in the cortisone-treated or thyroidectomized rats as defined by increased eyeball displacement. However, if the amount of protruding eyeball is compared with the size of the head, as represented by head weight, one observes that there is significantly more eyeball protruding

TABLE 2. EFFECT OF CORTISONE TREATMENT AND THYROIDECTOMY ON THE SIZE OF THE ORBIT AND THE EYEBALL PROTRUSION[a]

Group	Age (days)	No. of rats	Orbit		Protrusion of eyeball		
			Volume (ml.)	"mass"[b] (mg.)	Mg.	% of eyeball	% of head weight
I. Normal	28	6	0.184±0.004	202± 4	78± 9	56.0±6.6	1.26±0.14
II. Sham-operated	68	8	0.364±0.016	400±17	191±11	84.5±4.4	1.47±0.08
III. Cortisone-treated	68	7	0.228±0.010	251±11	156± 9	77.2±4.2	1.87±0.12
IV. Thyroidectomized (complete)	68	11	0.223±0.008	245± 8	165± 8	78.6±3.6	1.83±0.09
V. Thyroidectomized (incomplete)	68	5	0.300±0.016	330±16	160± 8	73.4±4.2	1.47±0.10

[a] Mean ± standard error. These animals are the same as those presented in Table 1.
[b] Orbit "mass" is an estimate of the amount of tissue which could be contained within the orbit, as determined by orbit volume × density (1.10).

relative to head size in the cortisone-treated (1.87%) and completely thyroidectomized (1.83%) than in the sham-operated rats (1.47%; $P<0.02$). The visual impression of this relationship is that the eyes are more prominent in the animals with the smaller heads. Although the eyeball/body weight ratio was larger in the 28 day old than in the 68 day old normals, the eyeballs did not appear prominent because the percentage of the eyeball protruding and the amount of protruding eyeball per unit head weight were much smaller in the younger animals.

The data for the incompletely thyroidectomized rats were intermediate between those of the sham-operated and of the completely thryoidectomized rats (Tables 1 and 2).

The effect of cortisone and complete thyroidectomy on the skull is presented in Figure 3 and Table 3. Although the skull length and width (bimalar) was markedly reduced in the dwarfed animals, there was no significant difference in the interorbital distance (bifrontal width) and only a

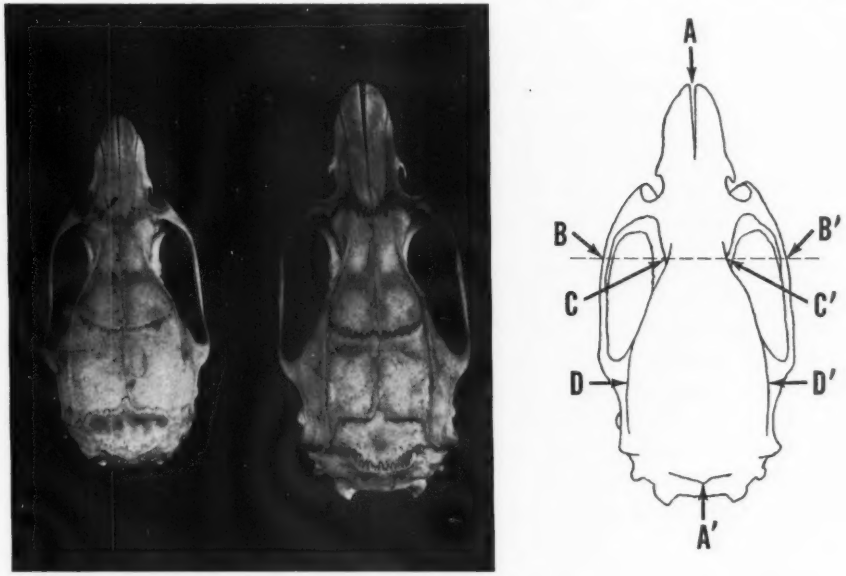

FIG. 3. The smaller skull was taken from a completely thyroidectomized rat (Group IV) and the larger from a sham-operated rat (Group II). The drawing at the right locates the points on the skull, designated by letters, which were used in making skull measurements presented in Table 3.

TABLE 3. EFFECT OF CORTISONE TREATMENT AND THYROIDECTOMY ON SKULL GROWTH[a]

Group	No. of rats	Skull length[b] (*A* to *A'*) mm.	Cranial width[b] (*D* to *D'*) mm.	Bimalar width[b] (*B* to *B'*) mm.	Bifrontal width[b] (*C* to *C'*) mm.	Eyeball diameter (estimated)[c] (*E*) mm.	$\frac{(C \text{ to } C') + 2(E)}{(B \text{ to } B')}$[d]
I. Sham-operated	3	41.5±0.2	15.3±0.2	19.8±0.1	6.4±0.1	6.0±0.1	0.93±0.01
III. Cortisone-treated	2	35.3±0.2	14.3±0.1	16.0±0.1	6.1±0.2	5.7±0.1	1.09±0.01
IV. Thyroidectomized (complete)	4	36.9±0.2	14.6±0.1	16.4±0.1	6.1±0.1	5.8±0.1	+1.08±0.01

[a] See Figure 2 for definition of skull measurements. These animals were taken from the groups presented in Table 1.
[b] Mean ± Standard Error.
[c] The diameter of the eyeball, which is a sphere in the rat, was estimated from the volume, determined by dividing the weight (average for each group (table 1)) by the density of the eyeball. The eyeball density, 1.04, was determined in several rats by measuring the mass and diameter.
[d] This ratio corresponds very closely to the ratio of the intercorneal to the intermalar dimensions as described by Sellers, Hill and Lee (1953).

slight difference in cranial width between the dwarfs and the sham-operated controls. The latter two dimensions probably reflect the size of the brain, the growth of which is unaffected by thyroidectomy (Scow and Simpson, 1945) and hypophysectomy (Walker *et al.*, 1950). It should be noted that the ratio in the last column of Table 3 was less than one in the normals and greater than one in the dwarfs even though this measurement did not include soft tissue. Since the bifrontal width and eyeball diameter were not appreciably affected by the treatment, this ratio was directly dependent on the skull width (bimalar).

DISCUSSION

Exophthalmos is defined as an abnormal protrusion of the eyeball from the orbit. In adult humans, this protrusion is the result of an increased mass of the retrobulbar tissues (Dobyns, 1950). The visual impression of clinical exophthalmos is that the eyeballs are more prominent than normal. In these experiments increased prominence of the eyeballs was observed in dwarfed rats even though there was no abnormal displacement of the eyeballs from the orbit. The eyeball prominence in this situation was due to an increase in the ratio of protruding eyeball mass to head size. This disproportion was primarily due to retarded growth of the skull and orbit without any growth retardation of the eyeball, and it is defined as apparent exophthalmos. Differentiation between apparent exophthalmos and exophthalmos as defined above is necessary because of the marked difference in the mechanism of the two.

In a recent study of the development of exophthalmos in propylthiouracil-treated rats (Sellers, Hill, and Lee, 1953), the presence of exophthalmos was determined by the ratio of the intercorneal distance to the distance between the malar processes. If this ratio was greater than one, the animal was considered to have exophthalmos. In their experiment, this

ratio was greater than one in nearly all the propylthiouracil-treated rats, but it should be noted that there was nearly a two fold difference in body weight between the treated and untreated rats. Since skull growth is markedly retarded and eyeball growth is unaffected in thyroid deficiency, as demonstrated in Table 3, it is necessary to ascertain what effect alterations in bone growth, especially in the bimalar width, have on this ratio if it is to be used to evaluate exophthalmos.

Normal eyeball growth, as measured by weight increase, has been observed in rats dwarfed by thyroidectomy at birth or weaning (Scow and Simpson, 1945), by hypophysectomy at five days of age (Walker *et al.*, 1950), and by cortisone treatment, as observed in the present paper.[2] It is possible that normal eyeball growth in the presence of retarded body growth would also occur in intact animals fed an inadequate diet, such as one deficient in protein, or in certain vitamins and minerals.

SUMMARY

Young rats dwarfed as the result of cortisone treatment or thyroidectomy developed an apparent exophthalmos. This was shown to be due to normal eyeball growth in the presence of marked inhibition of head and body growth. It was not related to any increase in mass of the retrobular tissues.

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[2] This disproportion between eyeball and body growth has also been observed in young thyroidectomized guinea pigs (Boas, 1953, unpublished) and in cortisone-treated chick embryos (Evans, 1953).

STUDIES ON WATER METABOLISM OF PYRIDOXINE AND PANTOTHENIC ACID DEFICIENT RATS

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IN PREVIOUS publications (Guggenheim and Hegsted, 1953; Guggenheim, 1954) it was reported that rats maintained on a diet deficient in protein or in thiamine exhibit a delayed diuretic response to a water load. Pitressin was found to exert a more powerful and longer lasting effect on deficient animals than on controls receiving a normal diet either *ad libitum* or in restricted amounts. Thiamine deficient rats injected with pitressin, excreted larger amounts of the hormone than did normal or pair-fed controls. Experiments carried out *in vivo* and *in vitro*, showed that the ability of the liver to inactivate pitressin was impaired in thiamine deficiency.

These observations raised the question as to whether the observed impairment of the ability of the liver to destroy pitressin and the consequential derangement of water metabolism were specific for the deficiency of thiamine or not. Studies were, therefore, undertaken in other types of experimental malnutrition. This paper presents the results obtained with pyridoxine and pantothenic acid deficient rats.

METHODS

Male rats were used throughout. Those subjected to pantothenic acid deficiency weighed 40 to 50 gm., and those employed in the experiments on pyridoxine deficiency, 150 to 170 gm. The controls were fed the normal 18% casein diet described previously (Guggenheim, 1954). Either pantothenic acid or pyridoxine was omitted from the diet of the experimental rats. In order to hasten the appearance of pyridoxine deficiency, 20 mg./kg. of desoxypyridoxine was incorporated into the diet of the animals kept on the pyridoxine free diet.

The hormone preparations used were: Pitressin (Parke, Davis Co.), desoxycorticosterone acetate (DOCA, Organon, OSS[1]), cortisone acetate (Cortone, Merck, Inc.), and ACTH (ACTHAR, Armour Laboratories). They were injected after dilution with saline.

RESULTS

Course of avitaminosis.—Five dietetic groups of rats were used. Group 1 received the normal diet *ad libitum*; group 2 was offered the pyridoxine deficient diet; group 3 was given the control diet restricted in amount to

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[1] DOCA was kindly supplied by Organon, OSS, Holland.

the level of intake of the pyridoxine deficient diet by the second group; group 4 received the pantothenic acid deficient diet, whereas group 5 was given the control diet restricted in amount according to the food intake of group 4.

Typical and severe signs of vitamin deficiency developed in the rats fed the pyridoxine deficient group after 4–5 weeks and in those fed the pantothenic acid deficient group after 8–9 weeks. At this stage of avitaminosis the rats and their pair-fed and *ad libitum* fed controls were taken for experiment. The weight of the pyridoxine deficient animals decreased during this period by 14 gm. (from 156 to 142 gm.) and that of their pair-fed controls by 4 gm. (from 171 to 167 gm.); the pantothenic acid deficient rats as well as their pair-fed controls increased in weight during two months by 55 gm. (from 44 to 99 gm., and from 49 to 104 gm. respectively). The control rats of group 1 increased their weight by 95 gm. during 5 weeks and by 140 gm. during two months (from 45 to 140 gm. and 185 gm. respectively).

Experiments with Pitressin.—The diuretic response of the avitaminotic rats to a water load and the effect of Pitressin were tested in the following way. A water load amounting to 8 per cent of the body weight was imposed on the animals. 30 minutes after the administration of the water load 50 mU. Pitressin in 0.1 ml. saline were injected subcutaneously or intrahepatically, and urine collection was begun and continued for 3 hours. The results of these tests are given in Table 1. The figures indicate per cent excretion of the administered water load.

It may be seen that the rats maintained on the pyridoxine or pantothenate deficient diet excreted significantly less urine during the first three hours after administration of a water load than did the control animals

TABLE 1. PER CENT EXCRETION OF WATER LOAD BY PYRIDOXINE AND PANTOTHENIC ACID DEFICIENT RATS

Diet	No. of tests	Pitressin	Means and standard errors		
			1 hr.	2 hrs.	3 hrs.
Normal	40	—	48 ± 2.0	67 ± 2.0	75 ± 1.9
Pyridoxine def.	44	—	24 ± 3.2	41 ± 3.6	46 ± 3.8
Pantothenic acid def.	30	—	33 ± 3.1	49 ± 2.7	56 ± 2.9
Caloric def.	20	—	46 ± 4.3	73 ± 4.5	81 ± 3.8
Normal	30	subcutan.	4 ± 0.8	12 ± 1.4	37 ± 3.2
Pyridoxine def.	11	subcutan.	5 ± 2.0	10 ± 2.5	38 ± 2.5
Pantothenic acid def.	10	subcutan.	4 ± 1.5	15 ± 2.9	44 ± 3.6
Normal	30	intrahepat.	7 ± 1.5	25 ± 2.1	52 ± 3.5
Pyridoxine def.	10	intrahepat.	5 ± 1.5	33 ± 4.2	56 ± 4.4
Pantothenic acid def.	11	intrahepat.	4 ± 4.4	29 ± 3.6	52 ± 2.8
*P**		—	0.02	0.001	0.001
Normal or caloric def. vs. Pyridoxine def. or Pantothenic acid def.		subcutan.	0.001 0; 0.7	0.3; 0.6	0.2; 0.8
		intrahepat.	0.4; 0.5	0.1; 0.3	0.5; 0

* Probability that the observed difference is due to chance.

TABLE 2. URINARY EXCRETION OF INJECTED ANTIDIURETIC HORMONE BY PYRIDOXINE AND PANTOTHENIC ACID DEFICIENT RATS

Per cent excretion of a water load by normal rats after injection of urine from Pitressin-treated rats. Means and standard errors

Diet of experimental	No. of tests	1 hr.	2 hrs.	3 hrs.
Normal	36	18 ± 2.2	45 ± 2.1	65 ± 2.0
Pyridoxine def.	11	12 ± 3.0	39 ± 5.6	60 ± 6.4
Pantothenic acid def.	13	15 ± 3.0	42 ± 4.3	63 ± 2.8

given a normal diet either *ad libitum* or in restricted amounts. (Since the response of the pair-fed animals of groups 3 and 5 to the water load was similar to that of group 1, the figures obtained with groups 3 and 5 are combined in Tables 1 and 4.) The response of the vitamin deficient animals to a water load seemed to be delayed. Pitressin, given either subcutaneously or intrahepatically, depressed the urine excretion in hydrated deficient animals to a similar extent as in the normal rats.

The urinary excretion of injected antidiuretic hormone and the *in vitro* inactivation of the hormone by liver were tested as described previously (Guggenheim, 1954). Table 2 shows that the rats injected with the urine of the deficient animals did not exhibit a greater antidiuresis than the animals treated with the urine of the control animals. Moreover, from Table 3 it is evident that rats injected with Pitressin which had been incubated with liver extract from pyridoxine or pantothenate deficient animals, did not show a stronger antidiuresis than rats which were treated with Pitressin after incubation with liver extracts from rats of the control group.

These experiments indicate that the delayed diuresis of pyridoxine and pantothenate deficient rats is not due, as in the case of thiamine deficiency, to a diminished ability of the liver to inactivate the antidiuretic hormone.

Experiments with adrenocortical hormones and with ACTH.—In order to elucidate the mechanism of the suppressed diuresis in pyridoxine and pantothenate deficiencies the following experiments were carried out. Avitaminotic rats and their controls were hydrated as described above and

TABLE 3. *In vitro* INACTIVATION OF PITRESSIN BY LIVER OF PYRIDOXINE AND PANTOTHENIC ACID DEFICIENT RATS

Per cent excretion of a water load by normal rats following injection of Pitressin previously incubated with liver extract. Means and standard errors

Diet of experimental rats	No. of tests	1 hr.	2 hrs.	3 hrs.
Normal	42	8 ± 0.9	39 ± 1.1	62 ± 2.5
Pyridoxine def.	36	7 ± 0.9	43 ± 2.4	64 ± 2.3
Pantothenic acid def.	24	6 ± 1.3	33 ± 3.6	0 ± 3.7

TABLE 4. THE EFFECTS OF DOCA, CORTISONE AND ACTH ON THE EXCRETION OF URINE IN HYDRATED PYRIDOXINE AND PANTOTHENIC ACID DEFICIENT RATS

Per cent excretion of a water load. Mean and standard errors

Diet	No. of tests	Treatment	1 hr.	2 hrs.	3 hrs.
Normal	12	DOCA	50 ± 1.8	53 ± 1.5	70 ± 1.5
Pyridoxine def.	12	DOCA	34 ± 5.3	43 ± 5.4	45 ± 5.6
Pantothenic acid def.	12	DOCA	30 ± 2.7	42 ± 3.7	49 ± 4.3
Caloric def.	6	DOCA	54 ± 2.0	68 ± 1.3	73 ± 1.5
Normal	18	Cortisone	51 ± 1.9	67 ± 1.8	72 ± 1.9
Pyridoxine def.	17	Cortisone	63 ± 2.4	71 ± 2.0	73 ± 1.8
Pantothenic acid def.	14	Cortisone	53 ± 3.9	63 ± 4.1	66 ± 3.8
Caloric def.	6	Cortisone	53 ± 5.8	79 ± 3.6	85 ± 5.6
Normal	12	ACTH	48 ± 3.0	73 ± 3.3	79 ± 3.5
Pyridoxine def.	13	Cortisone	39 ± 4.4	55 ± 4.1	60 ± 3.7
Pantothenic acid def.	13	ACTH	45 ± 4.5	67 ± 4.0	71 ± 4.0

subcutaneously injected with one of the following hormones: DOCA (1 mg. per rat, 60 minutes before water load), cortisone acetate (3 mg. per rat, 90 minutes before water load), and ACTH (4 mg. per rat, 90 minutes before water load). Urine was collected as described above. The results obtained are represented in Table 4.

It may be observed in Table 4 that DOCA is without any influence on water excretion in normal animals or in pyridoxine or pantothenate deficient rats and their pair-fed controls. Cortisone, however, which did not affect the urine excretion of the rats fed the normal diet either *ad libitum* or in restricted amounts, restored the diminished diuresis in the avitaminotic rats. The increased water excretion due to cortisone in these rats proved to be statistically significant. Similar results were obtained with ACTH. Whereas this hormone was without any diuretic effect in normal animals, it significantly increased the water excretion in both pyridoxine and pantothenic acid deficiencies.

It appears, therefore, that the diminished water excretion observed in pyridoxine and pantothenate deficient rats is due to a lack of cortisone-like substances. This lack does not result from an inability of the avitaminotic adrenals to produce or to secrete the hormone nor to an impaired response of the organism to the hormone. The underlying cause of the derangement seems to be rather a diminished production or secretion of the adrenocorticotropic hormone of the anterior pituitary.

DISCUSSION

Our studies demonstrate that rats in a state of pyridoxine or pantothenic acid deficiency show a delayed diuretic response to a water load. A similar phenomenon has already been described by us in protein and thiamine deficient rats (Guggenheim and Hegsted, 1953; Guggenheim, 1954). In

contrast to these latter nutritional disorders, the water retention in pyridoxine and pantothenate deficiency is not due to an impairment of the ability of the liver to destroy the antidiuretic hormone of the posterior pituitary. Experiments both *in vitro* and *in vivo* showed that the livers of rats suffering from pyridoxine or pantothenate deficiency inactivated Pitressin as fast as those of well nourished controls. Moreover, when avitaminotic rats were injected with Pitressin, they excreted this hormone as rapidly as did controls. Normal diuresis could be restored by cortisone or ACTH, whereas DOCA was without any effect. It may be concluded that the cause of the deranged water metabolism in these two types of nutritional disorder is a lack of production or secretion of cortisone-like substances due to an insufficient supply of ACTH. Our experiments leave open the question as to the lack of ACTH as a consequence of increased utilization of the hormone or a diminished production.

The role of adrenal cortical hormones in water metabolism is complex and is made the more confusing by the fact that the specific actions of these hormones vary greatly depending on the physiological background on which they operate. Gaunt and Birnie (1951) point out that there are two aspects to the action of cortical hormones on water metabolism which seem at first glance to be mutually contradictory: in some conditions they cause water retention and in other conditions they stimulate diuresis. In hydrated animals cortical hormones promote water loss; in this phenomenon cortisone and its relatives are distinctly more active than DOCA. Similarly, Ingle (1950) writes: "Although 11-desoxycorticosterone is more potent than any of the 11-oxysteroids in affecting electrolyte balance, it is less potent than cortisone in stimulating diuresis in acute tests and in preventing water intoxication." Recently Dexter and Stone (1952) observed an increased urine excretion in hydrated normal and adrenalectomized rats treated with cortisone. In our experiments neither DOCA nor cortisone or ACTH was found to affect water excretion in normal animals, but a pronounced diuretic effect of cortisone and ACTH was noted in pyridoxine and pantothenic acid deficiency.

A delayed diuresis in rats after a water load and its prevention by adrenal cortical hormones has already been described in deficiency of pantothenic acid (Gaunt, Liling and Mushett, 1948) and of pyridoxine (Stebbins, 1951a). Dumm and Ralli (1953) have noted a delayed diuresis following a water load in pantothenic acid deficient rats. It is interesting to note that in their experiments on adrenalectomized rats maintained on a high pantothenate diet, DOCA accelerated water excretion, whereas cortisone or ACTH did not.

Evidence of impaired adrenal function in deficiencies of pyridoxine (Stebbins, 1951b; Ershoff, 1952) and of pantothenic acid (Morgan, 1951;

Ershoff, 1952; Novelli, 1953) has been accumulated during recent years. Ershoff (1952) showed that pyridoxine deficient rats may have an impaired ability to produce the increased amounts of ACTH required during chronic stress. Although no impairment in the lymphopenic response to epinephrine occurred in pyridoxine deficient rats exposed to acute stress, a marked lack of response to chronic stress was observed in this deficiency.

Dumm, Ovando, Roth and Ralli (1949) found that the lymphopenia that normally occurs in rats following stress or injections of ACTH was largely abolished in pantothenic acid deficient rats. Cowgill, Winters, Schultz and Krehl (1952) tested pantothenic acid deficient rats with respect to both lymphocyte and eosinophile response to doses of ACTH and cortisone. Cortisone produced a marked drop in both of these blood elements, whereas ACTH induced a negligible eosinopenia and lymphopenia. In other experiments pantothenic acid deficient animals were given phloridzin. A striking decrease of urinary glucose excretion was noted, whether the rats were treated with ACTH or not. Cortisone, however, restored the glucose excretion to normal levels. Likewise, the impaired ability of deficient rats to deposit glycogen in the liver could be remedied by cortisone but not by ACTH. The authors concluded that the main defect in pantothenic acid deficiency occurs in the adrenals, but they consider also the possibility of a lesion in the anterior pituitary. In the experiments of Ershoff and coworkers (Ershoff and Parrott, 1953; Ershoff, Alfin Slater and Gaines, 1953) no impairment of lymphopenic response occurred in pantothenic acid deficient rats injected with ACTH but a marked impairment was observed after epinephrine administration. These findings suggest that inadequate synthesis or secretion of ACTH may have been responsible, at least in part, for the manifestations of adrenal dysfunction in pantothenic acid deficiency.

Our experiments do not contradict the well founded facts of an adrenal lesion in pyridoxine and pantothenate deficiencies, but they lend support to the view, at least as far as water metabolism is concerned, that this lesion is due to an inadequate stimulation of the adrenals by the adrenocorticotropic hormone.

SUMMARY

1. Rats maintained on a diet deficient in pyridoxine or in pantothenic acid exhibit a delayed diuretic response to a water load.

2. Pitressin evokes the same response in deficient rats as in well nourished controls. When pyridoxine or pantothenate deficient animals are injected with Pitressin, they excrete similar amounts of the hormone as do normal controls. Experiments carried out *in vivo* and *in vitro* showed that the ability of the liver to inactivate Pitressin is not impaired in these nu-

tritional disorders. Thus Pitressin was found not to be involved in this derangement of water metabolism.

3. DOCA, cortisone and ACTH were found to be without any effect on the diuresis of normal hydrated rats. Animals suffering from lack of pyridoxine or pantothenic acid increased their urine excretion after injection of cortisone or ACTH. DOCA had no influence under these conditions.

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THE ADRENAL CORTEX AND SULFUR METABOLISM: THE NON-EFFECT OF GLUTATHIONE ON ADRENAL ASCORBIC ACID

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PREVIOUS studies have shown that ACTH influences the non-protein sulfhydryl concentration of the adrenal cortex, and conversely, that the feeding of *l*-cystine to humans alters the urinary steroid excretion, thus presumably indicating a change in adrenal cortical activity (Goldzieher *et al.*, 1953). On the other hand, repeated experiments with the addition of glutathione to *in vitro* systems of adrenal tissue have failed to show increased steroid production or augmentation of the steroid-producing effect of ACTH (Savard *et al.*, 1950). Since there are various lines of evidence suggesting that glutathione and ascorbic acid may have some metabolic relationship, it seemed pertinent to inquire whether glutathione has any influence on the well known adrenal ascorbic-acid-depleting effect of ACTH.

MATERIALS AND METHODS

Hypophysectomized male rats weighing about 100 gm. were obtained from the Hormone Assay Laboratories of Chicago and used 20 to 24 hours after operation. Determinations were performed according to the ACTH assay method of Munson *et al.* (1948) except that the Roe and Kuether (1943) technique of colorimetry was used. Aqueous solutions of glutathione were prepared immediately before use, neutralized with sodium bicarbonate, and diluted to a concentration of 20 mg./ml. The intravenous injection of 0.5 ml. (10 mg.) was carried out 1½ hours before sacrifice of the animal. Thus, in those experiments where both ACTH and glutathione were injected, ½ hour elapsed between the administration of the glutathione and of the ACTH. ACTH from the same lot[2] was used throughout, being made up to a concentration of 1 U./ml. and stored at −20° C. Appropriate dilutions in saline were prepared immediately before use in such a way that the total amount to be given intravenously (0.25 or 1.0 milliunit) was contained in 0.5 ml. solution. All determinations on adrenal tissue filtrate were done in duplicate.

RESULTS AND DISCUSSION

The first experiment in Table 1 indicates clearly that glutathione itself, in this dosage, does not affect the concentration of adrenal ascorbic acid

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[2] ACTH, lot 212-103, was supplied through the courtesy of Armour and Company.

TABLE 1

Experiment No.	Treatment	Number of animals	Adrenal ascorbic acid concentration, mg./100 Gm ± std. error
I	None (control)	9	470 ± 10
	GSH (10 mg.)	9	473 ± 4
II	None (control)	5	459 ± 15
	ACTH (0.25 milliunit)	6	450 ± 18
	ACTH (1.0 milliunit)	5	403 ± 5
III	ACTH, 1.0 milliunit (control)	10	387 ± 16
	GSH (10 mg.), then ACTH (1.0 milliunit)	11	387 ± 13
IV	ACTH, 0.25 milliunit (control)	10	439 ± 17
	GSH (10 mg.) then ACTH (0.25 milliunit)	11	411 ± 23

within 1½ hours. This parallels some unpublished observations wherein we noted that *l*-cysteine in doses of 25, 50 and 100 mg. per 100 gm. body weight also failed to alter adrenal ascorbic acid. It differs, however, from the recent report of Carey *et al.* (1951), who administered glutathione (up to 400 mg. per 100 gm. body weight) to intact rats and guinea pigs and observed alterations of adrenal ascorbic acid and cholesterol. However, these investigations were carried out in intact, not in hypophysectomized animals, and would therefore seem to indicate only that large (and admittedly toxic) doses activate the pituitary-adrenal axis. Moreover, with somewhat smaller doses of the order of 25 and 100 mg./100 gm., the observed fall in the measured adrenal constituents was slight.

Experiment II was carried out merely to test the dose-response of our particular strain of rats. It appears that 0.25 milliunits is a subliminal dose while 1.0 milliunits produces an appreciable fall of ascorbic acid. Use of these two levels might then show whether glutathione can augment the response to a subliminal dose or whether it can alter (in either direction) the response to a substantial but still physiological stimulus. It was found in experiments III and IV that pretreatment with glutathione did not alter the response to ACTH in any perceptible fashion.

We may conclude, then, that the exposure of normal adrenal tissue (which already contains GSH) to additional GSH does not alter its reaction to ACTH as regards ascorbic acid depletion (or as regards steroid production, judging from the findings of others). That sulfhydryl is necessary for normal cortical function has been shown by Hofmann and Davison (1953), who removed the SH present in adrenal tissue homogenates by means of n-ethyl maleimide, and found that ACTH would no longer promote steroid production.

These observations leave unexplained the effect of cystine on urinary steroid excretion, unless one invokes qualitative alterations of adrenal secretion or the possibility that the metabolic pathway of the secreted steroids is changed in some way. Furthermore, as it appears that only a certain minimum quantity of GSH is necessary for the operation of the adrenal steroid-producing mechanism, a study of the rate of adrenal GSH turnover at various levels of adrenal function seams more promising than determinations of total GSH. Studies with radioactive GSH along these lines are now in progress.

SUMMARY

Intravenous glutathione administered to the hypophysectomized rat fails to alter the concentration of adrenal ascorbic acid, and does not affect the ascorbic-acid-depleting properties of ACTH.

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THE ROLE OF THE KIDNEY IN THE PARATHYROID CONTROL OF THE BLOOD CALCIUM AS DETERMINED BY STUDIES ON THE NEPHRECTOMIZED DOG

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DESPITE numerous studies, the mechanism of action of the parathyroid hormone remains a subject of controversy. The view which has received widest acceptance considers the primary action of the parathyroid hormone to be exerted by accelerating the excretion of phosphate through the kidney with secondary effects on the calcium level of the blood (Albright and Reifenstein, 1948; Crawford *et al.*, 1950; Milne, 1951; Tweedy *et al.*, 1950). Opposed to this view is a mass of data which indicates that the parathyroid hormone also exerts significant extra-renal effects, particularly on the bones (Barnicot, 1948; Collip, *et al.*, 1934; Jaffe, 1933; McClean, 1941; Monahan, 1944; Stewart and Bowen, 1952) which are responsible for its hypercalcemic action.

In order to dissociate the renal from the extra-renal effects of the parathyroid hormone, numerous studies have been performed on the nephrectomized animal. However, as indicated by Greep (1948) in his review of the subject, these experiments are for the most part vitiated by the abnormal state of the chemical composition of the blood induced by nephrectomy. The development of a procedure whereby the nephrectomized dog may be kept in good condition for periods of months (Grollman, Turner and McLean, 1951) afforded a means for avoiding the pertinent criticism of previous studies. The present paper harmonizes the conflicting deductions of earlier workers and permits certain definite conclusions regarding the role of the kidney in the mechanism of parathyroid action. In addition, studies have been made of the diffusibility of calcium, magnesium and inorganic phosphate across the peritoneal membrane.

METHODS

Mongrel dogs of either sex and variable size were nephrectomized in stages by an extraperitoneal approach through a lumbar incision. Sodium pentobarbital supplemented when necessary, by ether was used as an anesthetic for the first operation; ether alone was used for the removal of the second kidney. At least two weeks elapsed between the operations. The dogs were placed on a calorically adequate potassium-free diet for a few

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days prior to the second nephrectomy and were maintained on a carbohydrate-fat diet, free of electrolytes, with an amount of water (25 ml. per kg. of body weight) equal to their insensible perspiration following nephrectomy. Such animals survive for periods of 5 to 10 days instead of 2 to 4 days as is the case when they are starved or given access to their usual diet. By subjecting the animals to peritoneal lavage (Grollman, McLean and Turner, 1951) the disordered composition of the blood of such animals may be corrected and life prolonged for periods of several months. The procedure of the peritoneal lavage is described in detail elsewhere (Grollman, 1954).

In some of the dogs the thyroid and parathyroid glands were also removed, after the first nephrectomy. The completeness of the operation was demonstrated by allowing the animals to go into tetany. To avoid the complicating factor of thyroid insufficiency, the diet of these animals was supplemented by the addition of 20 mg. of dessicated thyroid daily.

Serum calcium was determined by Clark and Collip's (1925) modification of the Kramer-Tisdall method; serum magnesium by Hawk, Oser and Summerson's (1947) modification of Denis' method; inorganic phosphate by the method of Fiske and Subba-Row (1925); the Coleman Universal Spectrophotometer was used for all colorimetric comparisons.

EXPERIMENTAL DESIGNS AND RESULTS

(1) *The maintenance of the parathyroidectomized dog by means of peritoneal injections*

It is generally accepted that the early effects of parathyroidectomy are due to hypocalcemia which may be prevented by dietary methods which maintain the calcium concentration of the blood at its normal level. As shown previously (Grollman, 1952) the acute effects of adrenalectomy in the dog, which are due to aberrations in water and electrolyte balance, may be prevented by the daily injection intraperitoneally of a fluid having the composition of an ultrafiltrate of blood. The same ameliorative effects were observed in the parathyroidectomized dog, tetany being prevented readily by the peritoneal injection of this solution. The calcium concentration of the blood plasma under these conditions remained sub-normal but could be maintained at a normal level by increasing the calcium content of the peritoneal fluid by the addition of one gram of calcium chloride per liter.

The average results of three experiments are shown in Table 1. The well-known effect of thyroparathyroidectomy in depressing the serum calcium, elevating the inorganic phosphate and lowering the serum magnesium are shown in Experiment 2 as compared to the control values of Experiment 1. The tetany induced by thyroparathyroidectomy was relieved and the serum calcium level restored to normal levels by the daily injection intraperitoneally of a liter of solution having the composition of an ultrafiltrate of blood plasma enriched with calcium as shown in Experiments 3 and 4.

It will be noted from Table 1 that despite the correction of the hypo-

TABLE 1. THE AVERAGE FASTING SERUM CONCENTRATIONS OF CALCIUM, MAGNESIUM, AND INORGANIC PHOSPHATE IN NORMAL, THYROPARATHYROIDECTOMIZED AND NEPHRECTOMIZED DOGS. HYPOCALCEMIA FOLLOWED NEPHRECTOMY OF THE PARATHYROIDECTOMIZED DOG BUT NORMAL SERUM CALCIUM WAS MAINTAINED BY PERITONEAL INFUSIONS OF A "BLOOD-ELECTROLYTE" SOLUTION WITH ADDED CALCIUM CHLORIDE

The results are averages obtained on 3 animals

Expt. No.	Experimetal conditions	Calcium	Inorganic phosphate	Magnesium
		mg. %	mg. %	mg. %
1	Pre-operative controls	11.50	4.20	1.82
2	48 hrs. following thyroparathyroidectomy (in tetany)	6.77	6.33	1.62
3	24 hrs. later after peritoneal lavage	10.64	6.38	1.59
4	24 hrs. later	11.16	6.60	1.66
5	24 hrs. after nephrectomy	10.07	8.04	2.09
6	48 hrs. after nephrectomy	8.47	12.95	2.39
7	72 hrs. after nephrectomy (tetany)	6.96	17.25	2.42

calcemia by peritoneal injection, the hyperphosphatemia of hypoparathyroidism was not corrected by this procedure. This would indicate that the maintenance of a normal blood phosphate level is dependent upon a normal parathyroid function and is not simply incidental to the accompanying hypocalcemia. The hyperphosphatemia of hypoparathyroidism probably reflects a renal dysfunction in response to the absence of the parathyroid hormone.

(2) *The effect of nephrectomy in the parathyroidectomized dog*

Following nephrectomy (without peritoneal lavage) the blood serum calcium level in the thyroparathyroidectomized dog falls despite the concomitant rise in inorganic phosphate, with the recurrence of tetany as shown in Experiments 5, 6, and 7 of Table 1. These results confirm the findings of Monahan and Freeman (1944) who have also shown that the hypocalcemia of parathyroid deficiency is not dependent on abnormal losses of calcium or a reduced rate of excretion of phosphate by the kidney.

(3) *The effects of parathyroid extract following nephrectomy*

A crucial argument in support of the view that the parathyroid hormone exerts its primary effect through its action on the kidney is based on the observation that parathyroid extract induces little or no elevation in the calcium level of the blood in nephrectomized animals. Only when the extract is injected immediately after nephrectomy (Stewart and Bowen, 1951) does the typical rise in blood calcium ensue. This may be interpreted as indicating that (1) the primary action of parathyroid hormone is on the kidney; (2) the secondary effects of nephrectomy, particularly the disturbance in the composition of the blood induced by nephrectomy, prevents the hor-

mone from manifesting its effects in the uremic animal; or (3) that some incretory function of the kidney which is still present immediately following nephrectomy but diappears subsequently, is necessary for eliciting the effect of the parathyroid hormone. The experiments to be described demonstrate that it is the second of these possibilities, namely, the accumulation of phosphate and probably of other anions such as sulfate, citrate, *etc.*, which in the absence of the kidneys, prevents a rise in serum calcium level following the injection of hormone.

The reaction of dogs to the injection of 50 U.S.P. units of commercially available parathyroid extract per kilogram of body weight before and at varying periods following bilateral nephrectomy is shown in Table 2. It is evident from this Table that although parathyroid hormone exerts its usual effects in elevating the blood serum calcium on the day following nephrectomy, this capacity to react to the hormone is reduced thereafter concomitant with the rise in the inorganic phosphate and is practically absent on the sixth day following nephrectomy. When the animal was subsequently subjected to peritoneal lavage and the inorganic phosphate and other catabolites removed, the original capacity to react to parathyroid hormone was restored as shown in the last two experiments of the Table.

(4) *The Diffusibility of Calcium, Magnesium and Phosphate after Nephrectomy.*

The peritoneum offers an ideal membrane for the *in vivo* determination of the filtrability of the constituents of the blood, since this enters into equilibrium with the peritoneal fluid (Grollman, 1954). By comparing the concentration of a given constituent of the blood with its concentration in the peritoneal fluid after equilibration, one can determine its diffusi-

TABLE 2. THE EFFECT OF PARATHYROID EXTRACT (50 U.S.P. UNITS PER KILOGRAM OF BODY WEIGHT) ON BLOOD LEVELS OF NEPHRECTOMIZED DOGS AT VARYING TIMES FOLLOWING NEPHRECTOMY

All values are expressed as milligrams per 100 ml. of serum

Days following nephrectomy	Before injection of hormone			18 hrs. after injection of hormone		
	Ca	Inorganic phosphate	Mg	Ca	Inorganic phosphate	Mg
Pre-operatively	12.0	4.0	1.9	14.9	3.2	1.7
1	10.6	5.3	1.6	13.0	9.6	1.5
2	12.0	9.8	3.3	14.2	12.1	2.8
3	10.3	10.0	2.7	13.3	13.6	2.5
4	11.1	10.8	2.8	13.1	14.5	2.7
6	9.7	19.6	3.9	9.9	21.3	3.6
10*	10.5	7.4	2.0	13.2	9.4	1.8
15*	11.0	8.2	2.2	13.9	11.6	1.9

* Subjected to peritoneal lavage.

bility without use of an *in vitro* membrane, as has usually been done in the past. The state of binding is particularly important in the case of the ions involved in parathyroid function, since calcium and magnesium are bound to protein to a large extent. Inorganic phosphate is entirely free but in the lower animals (turtle, chicken) and in the mammal under abnormal conditions of hyperphosphatemia this ion too may be present in a non-diffusible form (Grollman, 1927).

In Table 3, the concentration of calcium, phosphate, and magnesium in the blood and in the peritoneal fluid at equilibrium are compared at widely varying concentrations of these ions. It will be noted that although the relative amount of diffusible calcium varies, a relatively large proportion of this ion is always present in a non-diffusible form. This is also true of magnesium. In the case of inorganic phosphate even under the conditions of the hyperphosphatemia of uremia, only a relatively small amount of phosphate is non-diffusible.

DISCUSSION

The present study demonstrates that parathyroid hormone acts directly on body stores of calcium (presumably the bones, but perhaps also on the tissues) to regulate the calcium level of the blood and that the hypercalcemic action of the hormone is not dependent on its renal action in increasing phosphate diuresis. This is proven by the demonstrated capacity of the hormone to cause hypercalcemia in the nephrectomized dog, even when the total inorganic phosphate of the blood is rising, as well as by the decrease in serum calcium and the induction of tetany in the parathyroidectomized-nephrectomized dog. The level of the inorganic phosphate, however, plays

TABLE 3. THE RELATION OF THE CONCENTRATION OF CALCIUM, MAGNESIUM, AND INORGANIC PHOSPHATE IN THE BLOOD AND PERITONEAL FLUIDS OF THE NEPHRECTOMIZED DOG

All values are expressed as milligrams per 100 ml. of serum

Calcium		Inorganic phosphate		Magnesium	
Serum	Peritoneal fluid	Serum	Peritoneal fluid	Serum	Peritoneal fluid
9.7	5.9	17.6	17.4	3.9	2.6
11.0	6.8	21.6	21.6	3.7	3.1
6.5	5.4	20.8	20.8	4.0	3.8
5.4*	4.0	18.2	18.2	4.4	4.3
10.6	8.2	6.9	7.8	1.7	1.4
10.7	8.5	15.4	15.4	3.5	3.1
14.9†	11.6	12.0	12.0	1.9	1.5
13.7	8.6	13.8	14.0	2.0	1.4
11.3	9.8	17.4	17.4	3.1	2.9
12.6	8.6	16.8	17.3	4.6	4.1
8.2	5.7	4.3	4.3	2.7	2.5
9.1	5.8	6.0	6.0	2.2	1.8

* Thyroparathyroidectomized and nephrectomized.
† After cortisone.

an important role in determining the relative rise in the blood calcium level following the injection of the hormone, this rise being inversely proportional to the level of the inorganic phosphate.

The present demonstration that the capacity of parathyroid hormone to mobilize calcium is not dependent on renal function is in accord with the conclusions of Ingalls *et al.* (1943); Stewart and Bowen (1951, 1942); O'Donovan (1951); Talmage *et al.* (1953) and others. The present study also explains the failure of Collip *et al.*, 1934; Neufeld and Collip, 1942; Tweedy *et al.*, 1950; and others to elicit a rise in calcium following nephrectomy by the injection of parathyroid hormone.

The failure of parathyroid extracts to elevate the serum calcium level under conditions of hyperphosphatemia in the nephrectomized animal may be attributed to the formation of insoluble calcium salts. Even normally, the product of $(Ca^{++})^3$ and $(PO_4^{--})^2$ exceeds their solubility product (Holt, La Mer and Chown, 1925). It is thus probable that in the nephrectomized animal where the inorganic phosphate and other anions which form non-dissociable and insoluble calcium salts are so greatly increased that these would be rapidly removed from the circulation by the reticuloendothelial system.

Although the immediate increase in the urinary excretion of inorganic phosphate which follows the injection of parathyroid extract may be an artifact, as shown by Stewart and Bowen (1952), there is reason to believe on the basis of many other studies, that the parathyroid hormone exerts an effect on the renal excretion of phosphate and that the homeostatic adjustment of the inorganic phosphate level of the blood may be mediated by alterations in parathyroid activity (Crawford, *et al.*, 1950; Milne, 1950). As in the case of other hormones (Grollman, 1947) the parathyroid glands probably exert an action at multiple sites in the body, at least on the bones as well as on the kidneys (Talmage *et al.*, 1953).

SUMMARY

The hypocalcemia and tetany which followed parathyroidectomy were effectively controlled by the intraperitoneal injection of an electrolyte solution having the composition of an ultrafiltrate of blood enriched with calcium.

Following nephrectomy of the thyroparathyroidectomized dog the blood calcium declined and tetany developed as in the animal with intact kidneys. The nephrectomized dog also responded by an increase in serum calcium level to the injection of parathyroid hormone. This response gradually diminished during the days following nephrectomy, but occurred even some weeks following nephrectomy if the animal were maintained by intermittent peritoneal lavage. It is concluded that the parathyroid hormone exerts

its effect on blood calcium by its direct action on the bones rather than through any action on the kidney.

The peritoneal membrane may be used as an *in vivo* membrane for determining the degree of binding of blood constituents. When studied by this method, calcium and magnesium are found to be bound to a considerable degree, but inorganic phosphate even at high levels appears to be almost entirely filterable.

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RATES OF DEVELOPMENT AND REGRESSION OF CHANGES DURING AND AFTER PROLONGED CORTISONE ADMINISTRATION IN RATS[1]

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THE effects of cortisone administration in producing morphological and biochemical changes in animals and man have been the object of considerable study during recent years (Ingle, 1950; Thorn *et al.*, 1953). However, the rate at which such changes are induced by prolonged cortisone administration, or the rate at which they regress upon discontinuation of treatment has received relatively little attention (Winter, Silber and Stoerk, 1950; Ingle, Prestrud and Nezamis, 1951; Sirek and Best, 1952). The present paper is concerned, therefore, with studying these aspects in greater detail, particularly at a dose lower than used in earlier studies, and with respect to parameters that have not previously been considered.

MATERIALS AND METHODS

Evidence is available that cortisone administration or adrenal cortical function may influence the levels of vitamin A in the serum and liver (Klopp, Tabor and Danish, 1951). It was therefore considered desirable to regulate the vitamin A intake in such a way that possible differences between the control and cortisone-treated animals might be more easily elicited. Accordingly, cortisone was administered during a period of vitamin A depletion, and the animals were restored to a normal vitamin A intake when the cortisone administration was discontinued.

Each of about 200 young weanling male Sprague-Dawley rats received a dose of 2000 I.U. of vitamin A and were then placed on the U.S.P. vitamin A-free diet. Four or five animals were tested at intervals for concentration of vitamin A in the liver until a value of about 70 I.U. per gm. was reached. At this point the remaining rats ranged in weight between 150 and 200 gm. They were divided into two approximately equal groups and continued on the vitamin A-free diet.

One group served as controls and received daily subcutaneous placebo injections of the vehicle in which the preparation of Merck cortisone acetate (henceforth called cortisone) was suspended. The second group received a daily subcutaneous dose of cortisone that was altered at various intervals so as to keep the weight of the rats generally at about two-thirds of the weight of the growing control animals during the 43-day period of administration. Thus, during the first 18 days the dose was 2 mg./day for the larger animals and 1 mg./day for the smaller. The dose was 1.5 mg./day for the 18th to the

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31st day and was then increased to 2 mg./day. In general, this dose averaged about 10 mg./kg. body weight/day.

At 15, 22, 31 and 43 days after the beginning of the injections 8 or 9 animals from each of the control and cortisone-injected groups were sacrificed, and the determinations, as shown in Figure 1 and Tables 1 to 4, were performed. The injections of cortisone and of the placebo and the feeding of the vitamin A-free diet were discontinued on the 43rd day. The animals were then placed on stock Purina Chow diet and subgroups of animals from each of the control and the cortisone-injected groups were sacrificed at 50, 64, 79 and 163 days. Twenty-four hours before sacrifice all animals were given 1 μc. of carrier-free I^{131} intraperitoneally, and food was withdrawn 18 to 20 hours before autopsy.

Sacrifice in all experiments was accomplished after an intraperitoneal injection of about 6.5 mg. of sodium pentobarbital in a volume of 0.1 cc. Blood was removed directly by aspiration from the dorsal aorta into a dry syringe. Adequate volumes of serum for vitamin A determinations were obtained by pooling, when necessary. Tissues were used immediately or transferred to a deep freeze, according to the type of determination to be done.

The following methods were employed: for serum vitamin A, Kimble's (1939); for kidney and liver vitamin A, Lewis *et al.* (1942); for serum ascorbic acid, Roe and Kuether's (1943); for thyroidal I^{131}, Money *et al.* (1951); for butanol-soluble I^{131}, Taurog and Chaikoff's (1946). Cholesterol of the adrenal gland was determined by the method of Schoenheimer and Sperry (1934) for serum; one of the glands was weighed, ground with sea sand (Reagent quality, Merck) and extracted overnight with 5 cc. of a 1:1 alcohol-acetone mixture. Analysis was performed on a 2 cc. aliquot of the centrifugate. The second adrenal gland was used for determination of ascorbic acid; the weighed gland was ground with sea sand and extracted with 12 cc. of 4% trichloracetic acid; the mixture was then shaken with Norite A. The analysis was performed on a suitable aliquot of the filtrate according to the procedure of Roe and Kuether (1943). For total nitrogen determination 1 cc. of a suitable dilution of the alkaline digest of the liver, which had been employed for the vitamin A determination, was digested with 1 cc. of 1:1 concentrated sulfuric acid-water mixture containing 0.25% $CuSO_4$.

RESULTS

The possibility existed that inhibition of overall growth due to cortisone administration might reflect itself in decreases of organ size which in turn might influence the content or concentrations of the various biochemical components. In order to elicit physiological significances from the data, it was decided to consider changes in the weight of organs both on an absolute basis and on the basis of 100 gm. of body weight. Similarly, changes in the biochemical components were considered on the basis of concentration, of total content in an organ, and of total organ content per 100 gm. of body weight.

Body Weight: Table 1 shows that, at the dosage schedule used, the cortisone-injected animals lost weight during the first 15 days, and then remained at about $\frac{2}{3}$ of the weight of the growing control groups. The average body weight of the cortisone-treated rats was significantly less than that of the control animals at each interval tested during the 43 days of cortisone

administration. When the cortisone was discontinued, the growth of the cortisone group accelerated, so that in the course of 120 days, the weight of the rats doubled and rose from 63% of the weight of the controls on the 43rd day of the experiment to 90% of the weight of the controls on the 163rd day. However, even at this point the difference between the two groups was significant.

Liver and Kidney Weights: The mean absolute weight of the liver in the cortisone-treated groups was not significantly different from that of the control groups during the first 31 days of treatment. At 43 days the mean absolute weight was significantly less than that of the controls. Upon discontinuation of the cortisone, the mean absolute weight continued to decrease until the 50th day of the experiment and was significantly less than the weight in the control animals until some time between the 79th and 163rd day of the experiment. The mean absolute weight of the kidney was in general significantly less in the cortisone-treated animals than in the control animals both during the period of cortisone administration and for the 120 days following its discontinuation.

A much more understandable pattern of changes in the liver and kidney weights evolved when these weights were calculated per 100 gm. of body weight (Table 1). On this basis, the liver and kidney weights in the treated animals had become significantly higher than those in the controls by the 15th day of administration and remained so throughout the period of administration. The liver weights, per 100 gm. of body weight in the treated animals, were about 40 to 50% higher than those in the control animals during the period of administration and returned to essentially normal

TABLE 1. CHANGES IN AVERAGE BODY WEIGHTS OF RATS AND OF WEIGHTS OF LIVER AND KIDNEY DURING AND AFTER CORTISONE TREATMENT

Duration of experiment (days)	Body weight				Liver weight as gm. per 100 gm. body weight			Kidney weight as gm. per 100 gm. body weight		
	Control	Cortisone-treated	Of cortisone-treated as per cent of controls	"t"†	Control	Cortisone-treated	"t"	Control	Cortisone-treated	"t"
a. During cortisone administration										
0	187 (8)*				3.55			0.80		
15	235 (8)	156 (8)	66	7.1	3.42	5.27	9.0	0.81	0.97	4.6
22	249 (8)	168 (8)	68	6.3	3.21	4.50	7.4	0.77	0.92	6.0
31	263 (8)	198 (8)	75	8.2	3.06	4.03	8.0	0.77	0.88	4.8
43	293 (8)	184 (9)	63	8.4	3.04	4.13	3.9	0.76	0.91	5.2
b. After cessation of cortisone										
50	310 (8)	177 (8)	57	10.1	3.13	3.46	1.8	0.78	0.88	2.8
64	331 (8)	238 (8)	72	8.1	3.16	3.37	1.4	0.74	0.77	1.2
79	328 (9)	277 (8)	82	2.4	3.14	3.07	0.4	0.79	0.76	0.6
163	369 (14)	332 (10)	90	3.2	2.58	2.67	0.3	0.72	0.68	1.6

* Figures in parentheses represent numbers of rats in each group and apply to same groups in subsequent tables.
† "t," in this and subsequent tables, represents evaluation of differences between control and cortisone-treated rats. A value for "t" >2 is considered as significant in the present work.

levels within 8 days after the discontinuation of the cortisone. The kidney weights, calculated in the same way, in the treated animals were about 20% higher than those in control animals during the period of administration, and returned to normal levels at some time between 8 and 22 days after discontinuation of the cortisone.

Adrenal and Thyroid Weights: Figure 1-a shows that the absolute weight of the adrenal glands decreased significantly and was about 50% of the control weight during the first 15 days of cortisone administration; it then remained at essentially the same fraction for the succeeding 28 days of cortisone administration. Upon discontinuation of cortisone administration, the absolute weight began to rise but still was significantly lower than that of the controls until sometime between the 79th and 163rd day of the experiment. The weight of the adrenals, on the basis of 100 gm. of body weight, did not decrease so markedly but was significantly less than the weights in the control group during the period of cortisone administration. When the cortisone was discontinued, the weight rose to 88% of the control weight by the 50th day and did not differ significantly from the control value on the 64th day.

In the present experiments the absolute weight of the thyroid was decreased significantly at the 15th and 22nd day of cortisone administration but not at the 31st or 43rd day. When cortisone administration was discontinued, the absolute weight decreased and remained significantly less than the control values throughout the entire period of observation. However, as may be seen from Figure 1-a, the thyroid weight per 100 gm. of body weight increased during the period of cortisone administration, being significantly higher than the control values at 31 days ($t=3.0$) and at 43 days ($t=3.9$). When the cortisone administration was discontinued, the thyroid weight per 100 gm. of body weight promptly dropped to the control values and remained there throughout the remaining period of observation.

Lymph Node and Thymus Weights: In the present study, the weight of the mesenteric lymph nodes decreased significantly and was 18% of the control values by the 15th day of treatment; it remained essentially at this level throughout the remainder of the cortisone administration (Fig. 1-b). After discontinuation the weight of the lymph nodes increased but did not attain parity with control lymph nodes until sometime between the 79th and 163rd day of the experiment. The weights of the lymph nodes were also decreased markedly when caluclated on the basis of body weight; however, the return to values of the same magnitude as the controls occurred somewhat more rapidly after discontinuation of the cortisone.

The weight of the thymus decreased significantly by the 15th day of cortisone administration to about 8% of the control values and remained at

TABLE 2. CHANGES IN LIVER NITROGEN DURING AND AFTER CORTISONE TREATMENT

Duration of experiment (days)	Liver nitrogen as per cent of liver weight			Liver nitrogen as mg. per 100 gm. of body weight		
	Control	Cortisone-treated	"t"	Control	Cortisone-treated	"t"
			a. During cortisone administration			
0	3.68					
15	3.46	3.06	3.4	122	161	9.6
22	3.75	3.54	1.5	120	161	7.8
31	3.29	3.52	2.9	101	141	9.0
43	3.69	3.80	1.3	112	157	5.0
			b. After cessation of cortisone			
50	3.77	3.86	0.8	117	134	3.8
64	3.81	3.76	0.6	120	127	1.5
79	3.75	3.54	3.3	117	109	1.0
163	3.83	3.89	0.7	99	105	2.0

this low level throughout the rest of the cortisone administration. After discontinuation on the 43rd day, the weight began to rise, but remained significantly less than the control values until some time between the 79th and the 163rd day of the experiment. The rise was more rapid when calculated on the basis of body weight.

Liver Nitrogen: Since the nonprotein nitrogen content of the liver is only a small fraction, about 10% or less, of the protein nitrogen (Ayres and Lee, 1936), it was decided to use the total liver nitrogen as a relative but convenient measure of protein storage. Table 2 shows that the concentration of liver nitrogen, expressed as per cent of wet weight of the liver, showed no particular trend either during or after cortisone treatment. However, when the total nitrogen content of the liver was expressed as mg. per 100 gm. body weight, there was a marked and significant elevation in this parameter in the cortisone-treated rats. When the cortisone was discontinued, the liver nitrogen content, expressed in the same manner, remained elevated until some time between the 50th and 65th day of the experiment, and then returned to control levels.

Adrenal Cholesterol and Ascorbic Acid: The administration of cortisone reduced the adrenal cholesterol concentration until it reached a level of 45% of the comparable controls on the 22nd day (Fig. 1-c). It remained essentially at this level for the duration of the injection period. When the cortisone was discontinued, the concentration did not begin to rise until after the 50th experimental day. It was still significantly less than the control value on the 65th day and also less than the control values, though not significantly so, during the remainder of the observation period. The *total* cholesterol content of the adrenal glands, calculated on an absolute basis

or per 100 gm. of body weight, decreased more markedly as a result of cortisone administration. The total content calculated on an absolute basis decreased to 23% of the control values by the 22nd day. After discontinuation of cortisone, the total cholesterol content increased slowly towards control values (Fig. 1-c).

The decreases in the concentration, total amount, and total amount of adrenal ascorbic acid per 100 gm. of body weight as a result of cortisone administration were significant and were, in general, parallel to, but less marked than, the changes in the adrenal cholesterol (Fig. 1-c). When the cortisone was discontinued, restoration towards normal levels did not begin until after the 50th experimental day.

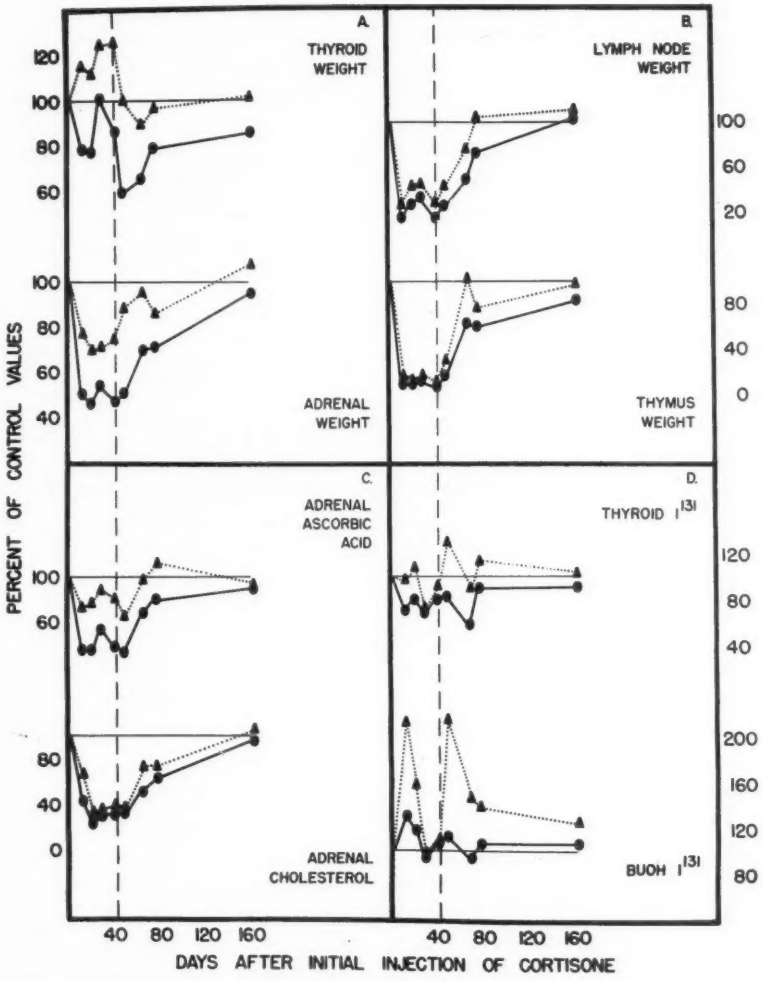
Serum Ascorbic Acid. Table 3 shows that the concentration of serum ascorbic acid rose significantly above control values between the 15th and 22nd day of the period of cortisone administration and remained significantly elevated until at least the 50th day of the experiment. By the 64th day of the experiment, the serum ascorbic acid concentration of the cortisone-injected animals had returned to control levels.

Collection of I^{131} by Thyroid: Figure 1-d shows that in the present study the thyroidal collection of I^{131} was decreased markedly throughout the period of cortisone administration. The decreases were all statistically significant; with the cessation of the cortisone administration the thyroidal collection of I^{131} rose to the levels of the control animals. When the collection was calculated per milligram of thyroid weight, there was no statisti-

TABLE 3. CHANGES IN SERUM ASCORBIC ACID CONCENTRATION DURING AND AFTER CORTISONE TREATMENT

Duration of experiment (days)	Concentration of serum ascorbic acid			"t"
	As mg. per 100 cc. in		In cortisone-treated rats as per cent of controls	
	Controls	Cortisone-treated		
	a. During cortisone administration			
0	1.18 (4)*			
15	1.16 (4)	1.08 (3)	93	0.8
22	0.87 (4)	1.42 (3)	163	3.8
31	0.80 (4)	1.81 (3)	226	6.6
43	0.81 (4)	1.54 (4)	190	6.9
	b. After cessation of cortisone			
50	1.02 (6)	1.33 (5)	140	7.7
64	1.15 (7)	1.17 (3)	102	0.3
79	1.13 (7)	1.01 (6)	89	1.8
163	1.15 (14)	1.21 (11)	105	1.1

* Figures in parentheses represents number of pools obtained from each group of rats.



● Represents values calculated from absolute weights of thyroid, adrenal, lymph nodes and thymus; from concentrations of adrenal ascorbic acid and cholesterol; and from I^{131} per whole thyroid. ▲ Represents values calculated from weights per 100 gm. of body weight; from total ascorbic acid or cholesterol in the adrenal gland; and from I^{131} per mg. of thyroid.

cally significant difference between the cortisone-injected and the control animals either during or after the period of administration. The butanol-soluble fraction of the thyroidal I^{131} was significantly elevated during the first 28 days of the cortisone administration, but then decreased to normal levels for the remainder of the period of cortisone administration and after cortisone was discontinued.

Vitamin A Metabolism: As has already been noted earlier the control and the cortisone-treated animals were placed on a vitamin A-free diet during the period of cortisone injections and were replaced on a vitamin A-containing diet when the cortisone was discontinued. This was done in order to gain information concerning the effect of cortisone on vitamin A metabolism at different tissue and blood levels of this vitamin, as well as on the process of depletion and repletion of vitamin A in the tissues and blood.

The concentration of vitamin A in the serum of the control rats decreased markedly during the first 43 days of the experiment (Table 4) on the vitamin A-free diet. Cortisone did not appear to influence significantly the rate of this decrease; only one of the mean values in the cortisone-injected animals (at 31 days) was significantly higher than the control value. When the cortisone was discontinued and the animals were placed back on a diet containing vitamin A, the serum concentrations rose to normal levels within 8 days, and neither at that time nor later were there any significant differences in the concentration of serum vitamin A between the control and the cortisone-injected animals.

The administration of cortisone did not appear to affect the storage of vitamin A in the liver. Thus, calculation of the concentration, as I.U. vitamin A per gm., showed no significant differences between the control and

TABLE 4. CHANGES IN CONCENTRATIONS OF VITAMIN A IN LIVER, KIDNEY, AND SERUM DURING AND AFTER CORTISONE TREATMENT

Duration of experiment (days)	Concentration of vitamin A as I.U./gm. in liver in				Concentration of vitamin A as I.U./gm. in kidney in				Concentration of vitamin A as I.U./100 cc. in serum in			
	Controls	Cortisone-treated	In cortisone-treated as per cent of controls	"t"	Controls	Cortisone treated	In cortisone-treated as per cent of controls	"t"	Controls	Cortisone-treated	In cortisone-treated as per cent of controls	"t"
					a. During cortisone administration							
0	73				13				116 (4)*			
15	38	38	102	0.1	14	3.7	26	4.8	138 (4)	149 (3)	108	0.6
22	19	16	84	0.4	16	3.5	22	4.4	127 (4)	142 (3)	112	1.3
31	13	7	54	1.5	7.8	2.6	33	3.2	84 (4)	115 (3)	135	4.1
43	3.1	1.2	39	1.9	2.3	0.9	39	1.4	35 (7)	34 (4)	97	0.1
					b. After cessation of cortisone							
50	151	159	105	0.8	5.3	2.5	48	4.3	82 (6)	95 (5)	116	1.3
64	380	425	112	1.7	6.4	4.7	74	2.2	100 (7)	95 (4)	95	0.4
79	637	614	97	1.0	5.4	4.6	86	1.3	89 (7)	78 (6)	88	1.2
163	1113	1187	107	1.5	3.4	3.8	110	1.2	74 (14)	70 (12)	95	0.6

* Figures in parentheses represent numbers of pools obtained from each group of rats.

the cortisone-injected animals at any stage of the experiment (Table 4). The total store was significantly less in the cortisone-treated animals at the end of the cortisone administration and at some points in the recovery period, but undoubtedly this was a reflection of the smaller absolute size of the liver in these animals. When the total liver vitamin A store was calculated per 100 gm. of body weight, there were no significant differences between the control and the cortisone-injected animals at any time during the period of administration.

In contrast to the above results, cortisone administration caused a marked decrease in the concentration of vitamin A in the kidney (Table 4) to about 20 to 40% of the control values. Upon discontinuation of the cortisone, the kidney vitamin A concentration in the cortisone-injected rats continued to be significantly lower than that in the control animals until some time between the 64th and 79th day of the experiment. The effect of cortisone on kidney vitamin A was just as evident when calculations were made on the basis of total storage of vitamin A in the kidney, or total storage per 100 gm. of body weight. In the normal control animals, the concentration of kidney vitamin A was highest at the beginning of the depletion period when the liver stores were moderate, and it was low when the liver stores were very low at the end of the vitamin A depletion period or when the liver stores were very high at the end of the repletion period.

DISCUSSION

The extent and rate of decrease of the weight of the adrenal gland and of its cholesterol and ascorbic acid concentrations during cortisone treatment, and their rate of restoration to normal values when the treatment was discontinued were in general agreement with those reported by Winter, Silber and Stoerk (1950). The extent of the decrease in the weight of the thymus during cortisone treatment was also about the same as that reported by these investigators. However, the rate of return of the thymus weight to normal levels, when calculated on an absolute weight basis, was slower than that reported by these investigators but quite prompt when calculated per 100 gm. of body weight. Changes in the size of the lymph nodes were not studied by Winter and his associates, although they observed that the weight of the spleen was reduced during cortisone treatment and restored to control values upon its discontinuation. The data in the present paper show similar but more marked effects on the lymph nodes.

In some respects the results in the present study differ from those reported by Winter and his associates (1950). These investigators found that administration of cortisone for six weeks had no effect upon the size of the liver or kidney when, presumably, the cortisone-treated animals were compared with controls of the same body weight. Our data show that the

weights of these organs, when calculated per 100 gm. of body weight, were significantly increased during cortisone treatment and returned rapidly to normal control values upon discontinuation of treatment.

That the size of the liver, as judged by weight, is increased as the result of cortisone administration for several days has been previously noted in the case of intact rabbits (Lowe, Williams and Thomas, 1951; Timiras and Koch, 1952), and in adrenalectomized rats (Silber and Porter, 1953). The increase in liver size appears to be due largely to hypertrophy of the parenchymal cells (Timiras and Koch, 1952). Harris and his associates (1953) have recently found that the kidney weight was increased significantly in guinea pigs after daily doses of 7.5 mg. or 15 mg. of cortisone for 10 days.

Winter *et al.* (1950) reported a small and equivocal rise in the size of the thyroid during cortisone treatment. The present data show that the cortisone-injected animals had significantly higher thyroid weights per 100 gm. of body weight during the cortisone treatment, with a prompt return to control levels upon cessation of cortisone administration. That this increase in thyroid size was not due to vitamin A depletion is, of course, evident from the fact that the controls were on a similar viatmin A-free diet as well as from earlier reports that level of vitamin A intake does not affect thyroid size (Money *et al.*, 1952).

The increased thyroidal collection of I^{131} during cortisone treatment paralleled the increase in weight of the thyroid, and no significant changes were as a whole manifest when the thyroidal calculation was calculated per mg. of thyroid. The butanol soluble I^{131}, representing mainly thyroxin iodine, showed significant increases as well as normal levels during both the cortisone and recovery periods. These irregular results may be due to the inherent error of the method.

Winter *et al.* (1950) reported that there was no change in the liver nitrogen during prolonged cortisone treatment. However, Silber and Porter (1953) found that, when the liver nitrogen was expressed, not as a fraction of the liver weight, but as mg. per 100 gm. of body weight, cortisone caused a marked increase in both the liver protein and nonprotein nitrogen. Clark (1953), employing the administration of a test dose of N^{15} labeled glycine to rats that had received cortisone for several days, also noted the same effect. Our data are in agreement with these observations. According to Clark, cortisone inhibits overall body synthesis of protein, and the liver acts as a detoxicating agent by preventing the accumulation of nitrogenous substances not elsewhere utilized in the body.

The finding in the present study that the concentration of ascorbic acid in the serum was elevated significantly is similar to that obtained by Booker and his associates (1951) after a daily treatment of rats with 2 mg. of cortisone and 10 mg. per kg. of ascorbic acid for 4 days. These investi-

gators found that cortisone decreased markedly the storage of ascorbic acid in tissues such as brain, muscle and fat and increased its urinary excretion. It is probable that the elevations of serum ascorbic acid observed in the present study in the cortisone treated animals represent a mobilization from the tissues.

The distribution of vitamin A in the various tissues of the rat depends upon a number of factors such as age, sex, and level of vitamin A intake. In the adult well nourished rat the liver contains about 90 to 95% of the total body stores of vitamin A, whereas the kidney contains only a few per cent (Clausen, 1942; Eden and Moore, 1951). However, in younger rats at low or moderate intakes, the kidney vitamin A may represent a sizable fraction of that present in the liver and under certain conditions may even exceed it (Eden and Moore, 1951).

The effect of hormones upon those aspects of vitamin A metabolism which are revealed in tissue storage and blood levels has received scant attention. On comparable vitamin A intakes the female rat has higher concentrations and storage of hepatic vitamin A than the male rat (Callison and Knowles, 1945; Brenner *et al.*, 1942; Moore, Sharman and Ward, 1951). Castrated male rats show a higher storage of vitamin A in the liver than normal controls. The concentration of plasma vitamin A is increased in men receiving testosterone and is decreased in bilaterally adrenalectomized rats (Klopp, Tabor and Danish, 1951).

The effect of hormones on the concentration of storage of vitamin A in the kidney is of especial interest. A curious bell-shaped relationship exists between the level of vitamin A intake and the concentration of vitamin A in the kidney of the normal male rat: the kidney vitamin A concentration is low at low intakes, increases to substantial values at moderate intakes, and *decreases* again markedly at high but not toxic intakes (Bodansky and Markardt, 1951; Moore, Sharman and Ward, 1951). This bell-shaped relationship is flattened out in the female rat. Thus, according to Moore and his associates (1951), whereas the normal *male* rat has kidney vitamin A concentrations of 2, 9, 38 and 15 I.U./gm. after daily intakes for about 4 to 6 weeks of 10, 20, 40 and 80 I.U., respectively, the normal *female* rat shows the following concentrations: 2.3, 8, 5, and 4 I.U./gm. It has previously been shown that a similar flattening of the relationship between kidney vitamin A concentration and the vitamin A intake is present in male rats treated daily with Reichstein's Compound L (Bodansky and Markardt, 1951). Although the present experiments were not carried out in the manner described above, a similar effect by cortisone is discernible. Thus the concentrations of kidney vitamin A in the control animals were quite high when the liver stores were moderate, as at the beginning of the vitamin A free period. When the liver stores were either very low, as at the end of the

vitamin A depletion period, or very high, as at the end of the vitamin A repletion period, the concentration of vitamin A in the kidney of the control animals was low. This relationship was not manifest in the cortisone-injected animals, since the kidney vitamin A was decreased at all stages of vitamin A depletion. We have at present no explanation for the bell shaped relationship between the level of vitamin A intake and kidney concentration of vitamin A in the male rat, nor any for the flattening out of this relationship in the female rat or by the administration of compound L or cortisone to the male rat.

SUMMARY

1. Cortisone was administered at a daily dose of 1 to 2 mg. for a period of 43 days to male rats weighing initially 150 to 200 gm. The weights of these animals remained at about two-thirds of a growing control group. Sacrifice of subgroups at intervals during cortisone administration revealed the following statistically significant changes in treated as compared with control animals: increases in liver, kidney and thyroid weights per 100 gm. of body weight; decreases in adrenal, lymph node and thymus weights on an absolute basis as well as per 100 gm. of body weight; increase in total nitrogen content of liver per 100 gm. of body weight; decreases in concentration of cholesterol and ascorbic acid per gm. of adrenal gland and in the adrenal contents of these compounds per 100 gm. of body weight; decrease of thyroidal collection of I^{131}.

2. The serum concentration of ascorbic acid was elevated significantly by the administration of cortisone, but that of vitamin A was not affected. The concentration of vitamin A in liver was not significantly altered in the cortisone-treated animals, but kidney vitamin A was markedly depressed.

3. After the cessation of cortisone administration both the treated and control groups were followed for an additional 120 days. The parameters discussed above reverted to the values of control groups at varying rates, but most of them attained parity within about 10 to 20 days after the end of the cortisone administration.

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UTILIZATION OF INJECTED L-ALANINE BY ADRENALECTOMIZED RATS[1]

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THE utilization of exogenous amino acids by fasted adrenalectomized rats has been studied by a number of investigators (Wells and Kendall, 1940; Evans, 1941; Samuels *et al.*, 1937; Lewis *et al.*, 1940) but interpretation of apparently contradictory results is complicated by the various procedures used. Cagan, Gray and Jensen (1950) administered casein hydrolysates intraperitoneally to adrenalectomized rats and found that there was a slower clearance of blood amino acids and urea than in normal animals but failed to find the expected increase in blood glucose. Moreover, unlike the case of the normal rats, the administered casein did not increase the liver amino acid oxidase of the adrenalectomized animals.

The problem of amino acid utilization by adrenalectomized rats has now been restudied. The results which are reported here were obtained from experiments in which L-alanine was used, and in which alanine alone was measured, instead of total amino acid nitrogen. Short time intervals were employed since the clearance is very rapid. The amino acid accumulates in the liver and kidney until it reaches a maximum concentration in 10–20 minutes after injection. Thus, by this procedure, it was possible to follow not only the disappearance of injected alanine from the blood, but is apparent utilization by the liver and kidney as well. By measuring other amino acids simultaneously, it was possible to detect transfers of the amino group, as shown by increases in the concentration of aspartic and glutamic acids. Blood keto acids were also determined and the results suggest that pyruvic acid is utilized to a greater extent in the adrenalectomized rat. L-alanine however, appears to be utilized to the same extent by both intact and adrenalectomized rats.

METHODS

Female[2] Wistar rats weighing approximately 200 gm. were used. In all instances the animals were fasted for 16 hours before injecting the alanine. Adrenalectomized rats

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[2] Few experiments were carried out with male rats. There was no difference in the results obtained.

were used after a minimum of one week rest following the operation; during this time salt was incorporated in the drinking water and the animals fed Purina pellets *ad libitum*. Each rat was given one milliliter of alanine solution containing 2000 micromoles; the solution was injected in the tail vein while the animal was kept under light ether anesthesia. After a given period of time (10, 20, 30, 45 and 60 minutes) the animals were sacrificed by a blow on the head, and a sample of blood removed from the heart. The liver and kidneys were removed as quickly as possible and aliquots weighed for analysis. Part of the blood and the liver and kidney samples were extracted by a previously reported method (Awapara, 1948). Alanine, glutamic acid, aspartic acid, glycine-serine and "under glutamic acid" were determined by paper chromatography using some recent modifications (Kit and Awapara, 1953). Total keto acids were measured by the method of Friedemann and Haugen (1943), with pyruvate as a standard.

RESULTS AND DISCUSSION

Six groups of adrenalectomized animals, including one control group which received saline (zero time) were used in these experiments. Similar series of intact animals served as controls. The values reported in Tables 1a, 1b, and 1c are the average values obtained from four experiments; we have omitted from the tables values obtained for "under glutamic acid" and for the glycine-serine component (the glycine and serine are measured together because they cannot be resolved completely) since they showed no change, after the administration of alanine. Of some interest is the fact that the liver of adrenalectomized rats contains invariably from two to three times more "under glutamic acid" than intact rats. This ninhydrin spot has been identified as containing mostly ethanolamine phosphoric ester (Awapara, Landua and Fuerst, 1950) but it is possible that arginosuccinate is also a component, (Ratner, Petrack and Rochovansky, 1953). No explanation for the increase of this component can be given at present.

From the tables one can easily see that alanine penetrates the kidney and liver effectively and in a short time, in both the adrenalectomized and intact groups. In both groups the observed maximum concentration occurs between 10 and 20 minutes; it is possible that the true maximum occurs sooner. In 60 minutes the concentration of alanine in the liver is much lower, approaching the resting value. At the end of one hour, the alanine concentration appears to be greater in the livers of the adrenalectomized animals, as compared to the intact animals. This does not happen in the kidney, where the concentration of alanine is about the same for both groups of animals at all points. The difference is probably fortuitous; we have repeated measurements of alanine 60 minutes after the injection but the results showed no real differences. The alanine measured at the end of one hour represents a small fraction of the alanine injected; in the liver it represents about 2 per cent. The values given in the tables suggest that if differences exist in the rates of utilization of L-alanine, the differences must be very small and escape detection by the procedures used here. In

TABLES 1a–c. UTILIZATION OF INJECTED L-ALANINE BY NORMAL AND ADRENALECTOMIZED RATS

(All values are given in micromoles per gram fresh tissue; each value is the average from four animals)

TABLE 1a

Liver

Time	Ala		Glu		Asp	
	C	Ad	C	Ad	C	Ad
0	1.6	1.3	1.0	1.3	1.0	1.3
10	22.4	17.7	4.8	4.9	4.8	4.9
20	19.7	18.6	4.1	5.0	4.5	5.0
30	13.9	13.0	4.1	5.7	3.7	5.7
45	10.1	11.0	3.0	3.8	2.8	3.8
60	4.5	8.3	2.3	3.7	2.0	3.7

TABLE 1b

Kidney

Time	Ala		Glu		Asp	
	C	Ad	C	Ad	C	Ad
0	1.0	1.3	5.3	5.3	1.0	1.4
10	13.6	14.8	6.0	5.9	1.6	1.6
20	8.7	10.3	6.4	6.2	1.8	1.5
30	5.0	6.0	5.0	6.8	1.6	2.1
45	3.4	3.9	6.0	6.5	1.7	1.8
60	3.0	3.5	4.6	5.8	1.7	1.8

TABLE 1c

Blood

Time	Alanine*		Pyruvate†	
	C	Ad	C	Ad
0			19.8	19.0
10	6.7	8.2	52.0	25.0
20	5.3	8.6	50.0	26.5
30	3.7	5.9	52.0	25.5
45	1.6	3.8	41.6	23.5
60	1.5	3.2	27.0	21.2

* In micromoles per milliliter of blood.
† In micrograms per milliliter of blood.

the liver itself, glutamic acid increases at first and then decreases slightly in both groups but the difference between the two groups is not impressive; the same applies to aspartic acid. The increase in the concentration of glutamic and aspartic acids in the liver, but not in the kidney, is probably related to transaminating activities of these organs; the alanine-glutamic acid transaminating activity of the liver (in rats) is known to be over four times greater than that of the kidney (Awapara and Seale, 1952). The

values in Table 1c indicate that alanine disappears from the blood of intact rats to a greater extent than from the blood of adrenalectomized rats. This is possible, but does not necessarily mean that alanine is better utilized by the intact animals. The amount remaining in the blood, is a small fraction of the amount injected; of the 2000 micromoles injected, less than 100 micromoles remain. Thus, a difference, however large in the amount remaining is insignificant when compared with the total amount. It would be unjustified to infer from these values that adrenalectomized animals use alanine at a slower rate than intact animals.

In the intact rat, the injection of alanine brings about an increase in blood keto acids; (presumably mostly due to pyruvate) but in the adrenalectomized rat, the increase is very small. After the intravenous injection of pyruvate itself, the accumulation and subsequent disappearance of keto acids in the blood is also less in the adrenalectomized rats: in 30 minutes after the injection of 2000 micromoles of sodium pyruvate, there was 24 μg./ml. in the blood of adrenalectomized, whereas in the blood of intact rats there was 47 μg./ml. (average values from six animals in each group).

The utilization of alanine might involve some peptide and protein synthesis and some renal excretion. It seems likely, however, that in the time involved, the bulk of the alanine has been deaminated and the resulting pyruvate disposed of through various channels. The results are consistent with the suggestion of Wells and Kendall (1940) that the limited capacity for glyconeogenesis of adrenalectomized animals is not attributable to an inability to utilize exogenous amino acids. When attention is directed to the difference in blood pyruvate, however, it is apparent that the further disposition of the three carbon compound may not be the same. Thus, the observations of Samuels (1936) and Lewis *et al.* (1940) might be related to the alternative pathways of utilization of the three carbon intermediate rather than to the deamination step.

Although the data reported here disagree with the results of Cagan, Gray and Jensen (1950) it is to be noted that comparison is not justified due to the use of casein hydrolysates by the authors cited and the fact that saline was withdrawn from the animals studied by them for three days prior to the experiment.

Further experiments with other glycogenic amino acids or known mixtures are indicated.

SUMMARY

L-alanine injected into adrenalectomized and intact rats was found to be utilized rapidly and to about the same extent by both groups. Most of the injected L-alanine disappeared from the blood, liver and kidney in about one hour. Increases in both glutamic and aspartic acid were observed and

the probable role of transaminases mentioned. Blood keto acids were found to increase in the intact rats but not in the adrenalectomized rats, 30 minutes after the injection of alanine. No great difference in blood keto acid concentration was found at the end of an hour.

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EFFECT OF ESTRONE, PROGESTERONE AND PITUITARY MAMMOTROPIN ON THE MAMMARY GLANDS OF CASTRATED C_3H MALE MICE

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SINCE the incidence of mammary cancer in the C_3H virgin may be as high as 97% in contrast to 0% in the male (Bittner and Huseby, 1946), a very important determinant would seem to be the hormonal factors influencing mammary growth. Many experiments have been performed in which estrogenic hormones in doses calculated to "feminize" the male mammary gland have raised the incidence of tumors to as high as 80% in males of this strain (Gardner, 1941). In the extensive literature of this field (Shimkin, 1945; Greenstein and Haddow, 1953) one may find reports on many tumor-inducing regimens employing different estrogens, dosages, methods of administration and periods of treatment. Only rarely have anatomical studies such as that of Trentin and Turner (1948) been made to determine the changes induced in the gland structure during the treatment period. And, insofar as we are aware, no one has worked out the shortest period during which the gland must be at a given developmental state in order that the tumor inciter be able to induce the neoplastic changes.

The following preliminary experiment, directed toward this end, has apparently fallen short in the time required for cancer induction; but it has given us some useful hormonal formulae capable of causing different degrees of mammary growth, in the C_3H male, corresponding to normal phases in the female gland.

EXPERIMENTAL

One-hundred-forty male C_3H mice, that had been nursed by their own mothers, were castrated at approximately six weeks of age and hormonal treatment was begun on the following day. The mice were divided into seven equal groups for different treatments and one animal from each group was sacrificed every 10 days and finally on D+45 (D = day of initial injection) five or six from each group were sacrificed in order to follow the progressive development of the mammary glands. Approximately 10 mice of each group were maintained for one year; and during that period were examined regularly for tumors. At necropsy the skin with adherent glands was stretched on cork and fixed

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overnight in 10% formalin. All glands from the year old mice and at least two from each of the others were dissected from the skin and stained in toto in alum carmine and cleared in methyl salicylate or embedded in plastic (Lyons and Johnson, 1953) for microscopic examination.

Each group received daily subcutaneous injections of hormonal combinations for 45 days as shown in Table 1. The controls (Group 1) received sesame oil, the vehicle for the steroids. In Groups 2, 4, 6 and 7 the estrone was injected for 10 days at the daily level of 0.5 μg. and increased to 2.5 μg. on D+11. Progesterone was given at the daily level of 1 mg. to Groups 3, 4 and 7 for 45 days. Mammotropin was injected into Groups 5, 6 and 7, as an alum precipitate at a 1 mg. daily dose for 45 days. This hormone has been shown to be strongly antigenic (Bischoff and Lyons, 1939) and symptoms referred to by Nelson *et al.* (1950) as anaphylactic were occasionally noticed and attributed to this protein. One mouse in each of Groups 6 and 7 died in shock.

RESULTS

The type and degree of mammary development attained after different intervals of treatment have been summarized in Table 1. Typical examples of the mammary glands of each group are shown in Plate 1. Since no tumors were found in any of the mice, even at the end of one year, it has been deemed important to report and illustrate the degrees of mammary development that did not predispose the C_3H male to tumor formation over the period studied.

Group 1: Sesame oil alone

Glands examined after 10, 20, 30, 40 and 45 daily 0.05 cc. subcutaneous doses of sesame oil showed no indication of proliferation or differentiation beyond the rudimentary duct system typical of C_3H males of this age. This stage of development is shown in Figure 4; and has been illustrated by Richardson (1951). Of the 10 mice sacrificed at the age of one year, or 9 months after the treatment period of 45 days, seven revealed only rudimentary male glands after a search of all of the mammary regions in the stained skin spreads. The other three mice showed glands comparable to those seen in Figures 1 and 2, and suggestive of an estrogen stimulation possibly of adrenocortical origin (Dorfman and Gardner, 1944; Greenstein, 1947).

Group 2: Estrone in sesame oil

Estrone was given subcutaneously in daily doses of 0.5 μg. in 0.05 cc. of sesame oil for 10 days; and then having shown by sacrificing one of the mice after this interval that little or no proliferation had occurred in the mammary apparatus, the daily dose was elevated to 2.5 μg. from D+11 to D+45. One mouse was sacrificed on D+10, D+20, D+30 and D+40; and six were sacrificed on D+45. Glands taken from these mice showed that a progressive lengthening and branching of ducts had occurred (Figs.

TABLE 1. MAMMARY CHANGES IN CASTRATED C_3H MALE MICE FOLLOWING DIFFERENT HORMONAL REGIMENS

Group	No. of mice	Autopsy D–D+45	Autopsy 1 year	Hormone	Daily dose		Mammary development							
					D–D+10	D+11–D+45	D+10	D+20	D+30	D+40	D+45	No. of mice	1 Year	No. of mice
1	20	10	10	Sesame oil	0.05 cc.	0.05 cc.	R*	R	R	R	R	6	R	7
													2D, CE	3
2	20	10	9	Estrone	0.5 μg.	2.5 μg.	D	D	2D	2D	2D, 2CE, AB	2	2D, CE	4
							CE	CE	2CE	3CE AB	3D, 3CE, AB SL	4	2D, CE, AB	5†
3	20	10	10	Progesterone	1.0 mg.	1.0 mg.	R	R	2D CE AB	R	R	6	R	6
													2D, CE	4
4	20	10	10	Estrone	0.5 μg.	2.5 μg.	D	2D	2D 3CE	3D 3CE	4D, 2CE 3AB, 2SL	4	3D, CE, AB	10
				Progesterone	1.0 mg.	1.0 mg.	CE	CE	AB	3AB 2SL	4D, 2CE, 3AB 3SL, LL	2		
5	20	10	9	Mammotropin	1.0 mg.	1.0 mg.	R	R	R	R	R	6	R	8
													D, CE	1
6	20	9		Estrone	0.5 μg.	2.5 μg.	D	D	2D	3D	AB, SL	3	2D, CE	2
				Mammotropin	1.0 mg.	1.0 mg.	CE	CE	CE AB	2CE 2AB, SL	3D, 3CE, 2AB SL, LL	2	2D, CE, AB	6
7	20	9	10	Estrone	0.5 μg.	2.5 μg.	D	3D	4D 3CE	4D 2CE	4D, CE, 4AB	5	3D, CE, AB	10
				Progesterone	1.0 mg.	1.0 mg.	CE	CE	2AB 2SL	4AB 3SL	3SL, 2LL			
				Mammotropin	1.0 mg.	1.0 mg.				2LL				

Key to abbreviations: *Duct development:* D slight, 2D moderate, 3D abundant, 4D most extensive.
Club ends: CE few, 2CE moderate number, 3CE many.
Alveolar buds: AB rare, 2AB moderate number, 3AB many, 4AB most numerous.
Small lobules: SL rare, 2SL moderate number, 3SL many.
Large lobules: LL rare, 2LL moderate number.

* Except where noted the glands of one mouse were examined at the specified period.
† The only hyperplastic nodule was found in one mouse in this group.

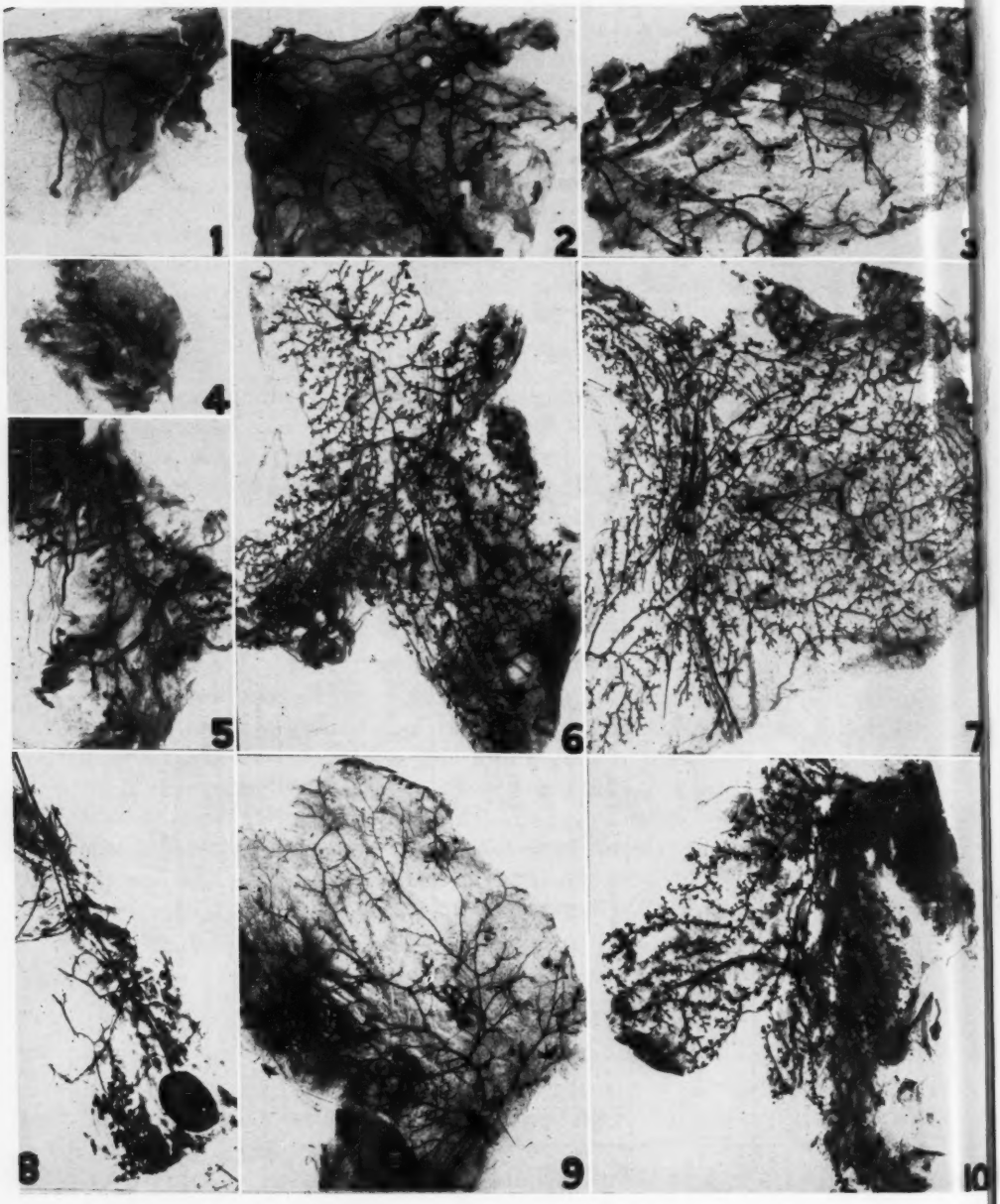

PLATE 1

1, 2, 3). Alveolar buds were observed by D+45 and in four of the six animals sacrificed on that day alveoli were found in small clusters, forming early, incomplete lobules. After the 45 day injection period and a total dose of 92.5 μg. of estrone the remaining 9 mice were saved and observed for 9 more months (age one year). There was no gross evidence of tumor in any of these animals. Mammary spreads showed persisting duct systems and scattered alveoli in five. A single hyperplastic "nodule" in one of these mice (Fig. 8) was comparable to one of the types described by Bittner and Huseby (1946), and others. Squamous metaplasia was not observed; but rather a small localized lobulo-alveolar growth differing but little from the generalized development of late pregnancy. Macrophages and lymphocytes had infiltrated the "nodule" just as they do in a gland regressing from a secretory or lobulo-alveolar phase of development.

Group 3: Progesterone alone in sesame oil

Daily 0.05 cc. (1 mg.) subcutaneous injections of progesterone were given for 45 days. Animals were sacrificed after the intervals already noted. Of all the mice sacrificed during the injection period only one showed mammary development beyond the rudimentary stage, such as seen in Figure 4. This mouse sacrificed on D+30 showed slightly larger ducts with a few alveolar buds. In the 10 mice in this group saved until they were one year old, no tumors were found; and only four showed glands developed beyond the rudimentary stage. These were different only in having a slightly more extensive duct system.

EXPLANATION OF PLATE 1

The mammary spreads were fixed in 10% formalin, stained in toto in alum carmine cleared in toluol and mounted in plastic. All are third mammary glands except the one shown in Figure 8, an inguinal gland. All are complete glands enlarged 4×. They are from castrated C_3H male mice treated as stated, beginning at the age of six weeks.

FIG. 1. 30 μg. estrone in 20 days.

FIG. 2. 55 μg. estrone in 30 days.

FIG. 3. 92.5 μg. estrone in 45 days.

FIG. 4. 5 μg. estrone and 10 mg. progesterone in 10 days. This is typical of the rudimentary glands of normal C_3H males, 1–3 months old.

FIG. 5. 55 μg. estrone and 30 mg. progesterone in 30 days.

FIG. 6. 55 μg. estrone and 30 mg. progesterone and 30 mg. M. H. in 30 days.

FIG. 7. 92.5 μg. estrone and 45 mg. of progesterone in 45 days.

FIG. 8. 92.5 μg. estrone in 45 days and then a lapse of 9 months without treatment. Note isolated lobular development (hyperplastic nodule) next to blot-like lymph node.

FIG. 9. 92.5 μg estrone and 45 mg. progesterone in 45 days. Then a lapse of 9 months without treatment.

FIG 10. 92.5 μg. estrone, 45 mg. progesterone and 45 mg. M.H. in 45 days.

Group 4: Estrone plus progesterone in sesame oil

The 20 mice in this group received 0.5 μg. of estrone plus 1 mg. of progesterone in 0.05 cc. of sesame oil subcutaneously for 10 days. A mammary gland taken from one of these mice on D+10 showed such poor development (Fig. 4) that the dose of estrone was increased as in Group 2 to 2.5 μg. per day until D+45. Glands taken from mice sacrificed on D+20, D+30 (Fig. 5), D+40, and D+45 (Fig. 7) showed that progressive duct and alveolar proliferation had occurred. By D+45 the glands showed development similar to that seen at about the tenth day of pregnancy. Small clusters of alveoli were uniformly distributed over a duct system with abundant arborization. No tumors were found in any of the 10 mice of this group saved until they were a year old. However, all of these animals showed partial maintenance of the duct system and also a scattering of mammary alveoli (Fig. 9).

Group 5: Mammotropin alone

Twenty mice were injected subcutaneously with a daily dose of 1 mg. in 0.1 cc. of an alum precipitate of sheep mammotropin. This hormone contained 25 I.U. per mg. and was injected in aqueous suspension at approximately pH 5.0 for 45 days. Mammary spreads made after the usual intervals showed no development beyond the rudimentary stage. Of the nine mice kept after this treatment until they were one year old, only one showed further mammary development and this was about equal to that seen in Figure 1. No evidence of tumor formation was found.

Group 6: Estrone in sesame oil plus mammotropin

The doses of estrone and mammotropin given to the 20 mice of this group were the same as used in Groups 3 and 5 respectively (see Table 1). The glands from mice sacrificed on D+10 and D+20 were essentially the same as those from the mice treated with estrone alone. By D+40 there had developed a duct system with moderate arborescence and small scattered alveolar clusters forming incomplete lobules. By D+45 the duct system was more extensive, but not as complex as in estrone+progesterone treated mice. However, such duct systems bore a few small lobules in all of the mice sacrificed on D+45.

Eight of the 20 mice of this group were saved and observed until they were one year old. They had received in 45 days of treatment a total of 92.5 μg. of estrone and 45 mg. of mammotropin. At no time did they show any evidence of tumor formation; and their mammary spreads showed only a partially maintained duct system with a scattering of alveolar buds.

Group 7: Estrone and progesterone in sesame oil plus mammotropin

The mice of this group received the same treatment as those of the preceding group plus 1 mg. of progesterone per day for 45 days. The steroids were injected together in 0.05 cc. of sesame oil and the M.H. was given separately as an alum-protein suspension.

Only rudimentary glands were seen in the mouse sacrificed on D+10. By D+20 there was an extensive duct system with a few alveolar buds and many club ends. The glands obtained on D+30 showed extensive and complex duct systems with a uniform distribution of alveoli many of which were in small clusters (Fig. 6). By D+45 most of the glands showed the same extensive duct formation with many small side branches and almost no club ends. Lobules were in different stages of completion, some being as large as those seen in the second half of pregnancy (Fig. 10). Some of the alveoli of these lobules showed accumulation of secretory material (prelactational) similar to that seen in glands at the end of pregnancy.

Ten mice of this group were saved until one year old, after having been injected with a total of 92.5 μg. of estrone, 45 mg. of progesterone and 45 mg. of mammotropin over a 45 day period. No evidence of tumor formation was seen at any time; nor were nodules found in any of the glands, all of which were inspected microscopically in the form of spreads. As in the estrone plus progesterone group these glands showed surprisingly well maintained duct systems with a sparse scattering of alveolar buds.

DISCUSSION

Summarizing the effects of the seven different hormonal regimens after 45 days, it may be said that sesame oil, progesterone, and M. H. (Groups 1, 4 and 5), administered alone induced no detectable changes in the rudimentary male mammary apparatus. Estrone alone or with M.H. (Groups 2 and 6) induced in the male gland a development similar to that of the virgin female or that seen in early pregnancy. The combination of estrone and progesterone with or without the addition of M.H. (Groups 3 and 7) changed a rudimentary gland to one comparable to that seen in mid-pregnancy, the best lobulo-alveolar growth seen in this experiment being obtained with the three hormones.

This provided three groups of mice with different degrees of mammary development to follow until they reached the age at which mammary tumors might appear. From Groups 1, 4 and 5, twenty-nine mice were saved for nine months following treatment; from Groups 2 and 6, seventeen mice; and from Groups 3 and 7, twenty mice.

No tumors were expected and none were found in the 29 mice from the groups in which treatment at the end of 45 days had induced no altera-

tion of the rudimentary glands. That three of the mice injected with sesame oil, one injected with M.H. and four injected with progesterone showed mammary development beyond the rudimentary stage, when examined at one year of age, was not surprising in light of the work on estrogen secretion by the adrenal cortex after castration (Dorfman and Gardner, 1944). It seemed reasonable to expect tumors in the remaining groups of mice since all of these had received sufficient hormonal treatment to induce mammary development equal to or surpassing that of the adult virgin, which in this strain has such a high mammary tumor incidence. With the one possible exception of a single small hyperplastic nodule in one gland of a mouse treated with estrone alone, no evidence of tumorigenesis was found at the end of a year.

It seems likely, then, that we have used as low a dose of estrone as might be expected to imitate in the C_3H male the ovarian out-put of the virgin, at least with regard to mammary stimulation. It is a dose level that permits the demonstration of synergism of estrone with progesterone and mammotropin. The period of 45 days during which these hormones acted upon the mammary gland may now be deemed shorter than the minimal time required by the tumor inciter to act upon an adequate substrate. Anatomically, exclusive of nipples, which are absent in male mice, the substrate in four of the groups was considered equal or better than that of the C_3H virgins for an estimated week before and after D+45. Therefore may be concluded that the mere attainment of a well developed mammary gland by male mice of a strain in which the mammary cancer incidence is high in the female and in which the virus factor is present does not constitute a sufficient prerequisite for tumorigenesis. The optimal period during which the C_3H male mammary gland must be adequately developed for tumorigenesis remains to be determined.

SUMMARY

The following conclusions were drawn after examination of the mammary glands of C_3H male mice castrated at six weeks of age and injected daily for 45 days as indicated.

1. No changes after total doses of 22.5 cc. of sesame oil (the vehicle for the steroids), or 45 mg. of progesterone, or 45 mg. of pituitary mammotropin.

2. Good duct and fair alveolar growth with the formation of a few small lobules after 92.5 μg. of estrone alone or combined with 45 mg. of pituitary mammotropin. The combination was slightly more efficacious than the estrone alone.

3. Extensive duct development, abundant alveolar growth, many small, and a few large lobules after 92.5 μg. of estrone plus 45 mg. of progesterone,

or after these hormones in combination with 45 mg. of pituitary mammotropin. With the latter regimen, in particular, it was possible to imitate in the male the mammary glands of the female in mid-pregnancy.

4. Regardless of the state of development attained after 45 days of treatment, no tumors were detected in any of the mice over a period of one year. Inspection of all mammary spreads of each mouse revealed only one small, hyperplastic nodule in an animal treated with estrone alone.

Acknowledgments

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EFFECTS OF PROTEIN ANABOLIC HORMONES ON RATES OF PROTEIN DEGRADATION AND PROTEIN LOSS IN THE FASTING DOG[1]

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NITROGEN storage induced in the adult female dog with either growth hormone (Bartlett and Gaebler, 1952) or testosterone propionate (Bartlett, 1953) is characterized by a higher rate of protein synthesis and a lower rate of amino acid catabolism than is observed in the same animal during nitrogen balance. The size of the nitrogen pool is increased during nitrogen storage induced with growth hormone (Bartlett and Gaebler, 1952) and is unaffected by testosterone propionate (Bartlett, 1953). Administration of growth hormone plus testosterone propionate induced marked nitrogen storage and gain in body weight in the adult female dog, yet produced no change in the rate of protein synthesis, decreased the rate of amino acid catabolism, and decreased the size of the nitrogen pool (Bartlett, 1953). This latter finding, which seemed of particular interest, was interpreted as being due to a decreased rate of protein degradation. In order to test this interpretation, we have extended our studies to the fasting animal. The rate of urinary excretion of nitrogen from intravenously administered N^{15} labeled glycine was determined in fasting bitches before and during treatment with growth hormone and testosterone propionate administered separately or together. From data obtained in this way, the specific rates of protein degradation and protein loss were calculated as described by Hoberman (1951).

EXPERIMENTAL

In general the animals were maintained as previously described (Gaebler, Bartlett, and Sweeney, 1951). Body weight and total urinary nitrogen excretion were determined daily, and, as soon as constancy of these measurements indicated that the animal was in nitrogen balance, a period of fasting was begun, food being withheld for 6 days. Glycine[2] labeled with N^{15} was administered intravenously in doses of 10 mg. per kilo body weight at the beginning of the second day of the fast, and excretion of the N^{15} label determined in the total urinary nitrogen during the subsequent 5 day period.

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[2] Labeled to the extent of 30 to 33 atom per cent N^{15}.

Rates of protein degradation and protein loss were calculated for the period between the fifth and sixth days of the fast, corresponding to the fourth and fifth days of hormonal stimulation. This period was chosen since it corresponded to that during which rates of protein synthesis and amino acid catabolism were determined in earlier studies on the kinetics of protein metabolism in the same animals (Bartlett and Gaebler, 1952, and Bartlett, 1953).

In experiments in which the effects of growth hormone or testosterone propionate on rates of protein degradation and protein loss were studied, the same schedule was followed and stimulation of the animal with the hormone begun when the N^{15} labeled glycine was administered. Growth hormone preparations were administered subcutaneously in doses of 5 mg. per day during the subsequent 5 day period and testosterone propionate intramuscularly at a level of 50 mg. per day for the 5 day period.[3] In the experiments in which growth hormone plus testosterone propionate were administered, the same levels of dosage and routes of administration were used.

RESULTS

In Table 1 are presented the data showing the effects of the different hormones on the excretion of glycine nitrogen by the fasting bitch. Excretion of the N^{15} label was very rapid during the first 24-hour period, reduced sharply during the second and third periods, and remained relatively constant during the fourth and fifth periods. Some variations in response were observed in the different dogs during the first 24-hour period following administration of labeled glycine and stimulation with hormone. For example, stimulation of Dog 44 with growth hormone, testosterone propionate, or, the combination of growth hormone with testosterone propionate, resulted in a higher percentage of the administered N^{15} being excreted during the first 24-hour period than during the corresponding period of the experiment in which the animal simply fasted. Dog 55 behaved similarly during the experiment in which the animal was stimulated with the combination of growth hormone and testosterone propionate. In all other experiments on Dog 55 and in all experiments on Dog 52, the percentage of N^{15} excreted in the urine of the fasting animal was reduced during the first 24-hour period of stimulation. In general, growth hormone, testosterone propionate, and growth hormone administered in combination with testosterone propionate reduced the percentage of administered N^{15} excreted in the total urinary nitrogen of the fasting dog during the second, third, fourth, and fifth days of hormonal stimulation.

[3] Armour's growth hormone preparation, 22 KR1, and Schering's Oreton (testosterone propionate) were used throughout the course of this study.

TABLE 1. EFFECTS OF GROWTH HORMONE, TESTOSTERONE PROPIONATE, AND GROWTH HORMONE PLUS TESTOSTERONE PROPIONATE ON THE EXCRETION OF GLYCINE NITROGEN IN THE FASTING ADULT FEMALE DOG

Hours after I.V. administration of N^{15} labeled glycine	Percentage of administered N^{15} excreted in the total urinary nitrogen of the fasting dog			
	Treatment			
	None	Growth hormone	Testosterone propionate	Growth hormone plus testosterone propionate
		Dog 52		
0– 24	30.9	25.3	25.7	21.9
24– 48	12.4	8.6	6.0	8.0
48– 72	8.1	4.8	4.1	4.0
72– 96	4.6	3.5	3.4	2.6
96–120	4.0	3.4	3.3	2.5
		Dog 44		
0– 24	20.8	25.5	23.5	28.2
24– 48	7.8	7.3	7.3	5.8
48– 72	4.9	4.8	4.1	3.7
72– 96	3.7	4.1	3.7	3.4
96–120	3.6	2.9	3.1	2.3
		Dog 55		
0– 24	23.2	19.8	20.6	23.8
24– 48	6.8	4.4	6.2	5.4
48– 72	4.2	2.6	4.1	5.4
72– 96	3.5	2.4	3.5	2.6
96–120	3.5	3.0	2.8	2.4

In Table 2 are tabulated rates of protein degradation and protein loss calculated from the N^{15} excretion data. The average rate of protein degradation for the three dogs was found to be 6.97 $(\pm 0.17)\times 10^{-3}$ per hour and the average rate of protein loss 3.03 $(\pm 0.70)\times 10^{-3}$ per hour during the fasting experiments. When such animals were stimulated with growth hormone, the average rate of protein degradation was reduced to 6.07 $(\pm 0.41)\times 10^{-3}$ per hour and the average rate of protein loss to 2.27 $(\pm 0.26)\times 10^{-3}$ per hour. Stimulation of the animals with testosterone propionate resulted in average values of 6.13 $(\pm 0.71)\times 10^{-3}$ per hour for the rate of protein degradation and 2.10 $(\pm 0.57)\times 10^{-3}$ per hour for the rate of protein loss. Stimulation of the animals with the combination of growth hormone and testosterone propionate resulted in average values of 4.43 $(\pm 0.28)\times 10^{-3}$ and 1.67 $(\pm 0.07)\times 10^{-3}$ respectively for rates of protein degradation and protein loss.

DISCUSSION

The observation that the percentage of administered N^{15} excreted in the total urinary nitrogen of the fasting adult female dog was in general decreased during the second, third, fourth, and fifth days of hormonal

stimulation seems particularly significant since this decrease occurred during the time when protein stores in the fasting animal are being mobilized to supply carbohydrates and to meet caloric and metabolic requirements in general. In respect to growth hormone, similar observations were made in some of the earlier studies in which crude anterior pituitary extracts of the hormone were used. Effects of the extracts in producing nitrogen storage were greatest when the protein catabolism was highest, as, for example, in the adult female dog showing a negative or slightly positive nitrogen balance during maintenance on a calorically adequate high protein diet (Gaebler, 1933, and Price, 1937).

The rate of protein degradation and protein loss data presented in Table 2, analyzed statistically by the methods of Fisher (1948) for small samples, indicate that although treatment of the fasting bitch with either growth hormone or testosterone propionate resulted in lower rates than were observed in the fasting animal, the differences were not significant.

TABLE 2. EFFECTS OF GROWTH HORMONE, TESTOSTERONE PROPIONATE AND GROWTH HORMONE PLUS TESTOSTERONE PROPIONATE ON RATES OF PROTEIN DEGRADATION AND PROTEIN LOSS IN THE FASTING ADULT FEMALE DOG

Dog	Rate of protein degradation (values $\times 10^{-3}$ per hour)	Significance*		Rate of protein loss (values $\times 10^{-3}$ per hour)	Significance*	
		t-value	*P*		*r*-value	*P*
			Fasting			
44	6.8			2.5		
52	7.2			4.0		
55	6.9			2.6		
Mean	6.97 ± 0.17			3.03 ± 0.70		
			Fasting stimulated with growth hormone			
44	5.5			2.3		
52	6.1			2.6		
55	6.6			1.9		
Mean	6.07 ± 0.41	2.84	0.02↔0.05	2.27 ± 0.26	1.44	0.2 ↔0.3
			Fasting stimulated with testosterone propionate			
44	5.7			2.3		
52	7.1			2.4		
55	5.6			1.9		
Mean	6.13 ± 0.71	1.61	0.1 ↔0.2	2.1 ↔0.57	1.45	0.2↔0.3
			Fasting stimulated with growth plus testosterone propionate			
44	4.2			1.7		
52	4.7			1.7		
55	4.4			1.6		
Mean	4.43 ± 0.28	10.28	<0.01	1.67 ± 0.07	2.73	0.05↔0.1

The ± values are standard deviations calculated according to Fisher (1948).
* *P* values less than 0.01 considered significant.

Treatment of the animals with the combination of growth hormone and testosterone propionate, however, resulted in a significant reduction in the rate of protein degradation. Rates of protein loss were not significantly altered in any case.

The significant reduction in the rate of protein degradation produced in the fasting dog stimulated with the combination of testosterone propionate and growth hormone supports the view that nitrogen storage and gain in body weight so induced in the adult female of this species is largely due to this shift in the kinetics of protein metabolism (Bartlett, 1953). Results of earlier studies (Bartlett and Gaebler, 1952, and Bartlett, 1953) and the present studies appear to indicate that an increase in the rate of protein synthesis and a decrease in the rate of amino acid catabolism are the major shifts in the kinetics of protein metabolism contributing to nitrogen storage and gain in body weight induced in the adult female dog with either growth hormone or testosterone propionate.

SUMMARY

Stimulation of the fasting adult female dog with growth hormone or testosterone propionate or a combination of growth hormone and testosterone propionate resulted in a reduction in the percentage of nitrogen of intravenously administered N^{15} labeled glycine excreted in the total urinary nitrogen during the second, third, fourth, and fifth days of hormonal stimulation. The rate of protein degradation was not significantly reduced by either growth hormone or testosterone propionate. Stimulation of the same animals with growth hormone plus testosterone propionate however, significantly reduced the rate of protein degradation. Reduction in the rate of protein loss, observed in all animals during stimulation with growth hormone or testosterone propionate or the combination of hormones, was not significant.

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THE EFFECT OF HYPOTHYROIDISM ON THE DEVELOPMENT OF EXPERIMENTAL UREMIA[1]

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CHANGES in hormonal balance affect the survival of animals subjected to experimental uremia. In general, those hormones exerting an anabolic effect increase the survival time of nephrectomized animals, while those promoting catabolism are detrimental to the uremic organism.

Bondy and Engel (1947) found that adrenalectomized-nephrectomized rats survived longer than the nephrectomized animal only when previously depleted of potassium. The terminal non-protein nitrogen was higher in the control than in the adrenalectomized group. It has also been found that the administration of desoxycorticosterone acetate prior to or after nephrectomy increased the survival time and decreased the rate of non-protein nitrogen rise (Seyle, 1940a, Selye and Nielsen, 1941; Dosne, 1941, Nylen, 1946; Rodbard, 1945; Masson, Corcoran and Page, 1949). The administration of adrenal cortical extract had no beneficial effect (Winkler, Smith and Hoff, 1942) or a definitely adverse one (Bondy and Engel, 1947) on survival following adrenalectomy. Testosterone administration has been reported to increase survival time of uremic animals (Masson, Corcoran and Page, 1949; Selye, 1940b) and apparently of man (Henderson, *et al.*, 1948). Mason, Corcoran and Page (1949) found desoxycorticosterone acetate and testosterone effective in increasing survival and lowering non-protein nitrogen only in the presence of a high carbohydrate diet, while in the presence of a sterile turpentine abscess, testosterone, desoxycorticosterone acetate, insulin, or high carbohydrate diet were without effect. Mirsky (1938) has shown insulin to have a definite anabolic effect on nephrectomized dogs.

Winternitz, *et al.* (1940) found a marked increase in survival time in one hypophysectomized-nephrectomized dog. A fibrous thyroid gland was demonstrated at post mortem examination. Thyroparathyroidectomized dogs subsequently prepared by these same workers did not survive nephrectomy longer than their controls. Hines and Freeman (1952) noted a longer survival time following bilateral nephrectomy, associated with a

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slower rate of change in the composition of the blood serum in thyroparathyroidectomized dogs than in their controls.

The present study was undertaken to compare the effects of thyroidectomy on survival time and certain constituents of blood in dogs and rats following nephrectomy.

MATERIALS AND METHODS

Nine dogs on a diet of commercial dogfood, bread and horsemeat, received subcutaneously a total of 9 millicuries of I^{131}.[2] This was injected in three separate doses of approximately 3 millicuries each. One month or more intervened between the last I^{131} injection and nephrectomy. Bilateral nephrectomy was performed through a mid-line abdominal incision using ether anaesthesia and an operative technique that observed aseptic precautions. The dogs were not fed, but were offered water *ad libitum* following nephrectomy. Serial blood samples were obtained for analysis from the 12 control and 9 thyroidectomized dogs.

Rats were discarded breeders of the Sprague-Dawley strain; a majority were males. Rats from each shipment acted as their own controls. Doses of I^{131} varied from a total of 3 millicuries to each animal (1 millicurie administered intraperitoneally every other week for a total of 3 doses) to 3.5 millicuries per kg. of body weight (Goldberg, *et al.*, 1950) administered in 1 dose. Those receiving a total of 3 millicuries I^{131} were fed commercial food[3] while those groups receiving a single dose of 3.5 millicuries per kg. of body weight received a diet low in iodine[4] for one month prior to I^{131} administration. One group was fed 0.1% 6-n-propyl-2-thiouracil in the commercial diet for 6 weeks prior to nephrectomy.

Another group of rats was given 10 mg. of 6-n-propyl-2-thiouracil per 100 gm. body weight in a 10% Gum Acacia suspension by stomach tube immediately after nephrectomy. One cubic centimeter of the suspension was equivalent to 20 mg. of propyl-thiouracil.

Blood samples were drawn from the abdominal aorta of rats under light ether anaesthesia 72 hours after nephrectomy.

Surgery on rats was done under ether anaesthesia without aseptic technique. The ureters were doubly ligated and sectioned or the kidneys were removed through a midline abdominal incision. No food was offered post-surgically but the animals were given distilled water *ad libitum*. The survival time was measured in hours from the time of operation to the time of death. For most rats it was accurate to within 1–2 hours. Data on the nephrectomized and ureteral ligated animals were pooled since no significant differences were found between the two groups.

Serum inorganic phosphorus was determined by the method of Kuttner and Lichtenstein (1925) with the modification of Gomori (1942).

Serum potassium was determined by means of a Beckman flame photometer. Nonprotein nitrogen was estimated on the Folin-Wu filtrate according to the method of Koch and McMeekin (Van Slyke and Hiller, 1949).

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[2] Obtained an allocation from the Atomic Energy Commission.

[3] "Lab Blox," product of Allied Mills, Inc., Chicago, Illinois.

[4] 20% fat, 20% vitamin-free casein, 8% Brewers yeast, 48% glucose, 4% Osborne-Mendel salt mixture (Osborne and Mendel, 1913) without added KI; Vitamins A and D were added.

TABLE 1. COMPARISON OF SURVIVAL TIME BETWEEN NORMAL NEPHRECTOMIZED AND THYROIDECTOMIZED-NEPHRECTOMIZED DOGS AND RATS

	Treatment	Number of animals	Mean survival (hours)	Standard deviation	*t*-ratio*
Rat	Control	52	88.13	15.1	
	Thyroidectomized (I^{131})	48	107.72	17.0	6.07
	Propylthiouracil (6 wks.)	4	123.0	4.95	4.50
Dog	Control	12	104.9	24.8	
	Thyroidectomized (I^{131})	9	182.8	47.8	4.86

* Youden, W. J., "Statistical Methods for Chemists," page 25, John Wiley & Sons, Inc., New York, 1951.

RESULTS

At necropsy, the thyroid of the dogs that received I^{131} varied from no grossly visible thyroid tissue to a mass of tissue about the size of a pea. The parathyroids were grossly intact. Each animal also had a marked increase in total cholesterol[5] following radiotherapy.

In some groups of rats there was slight gross evidence of any thyroid tissue remaining, which was further confirmed by microscopic sections. In those groups given a low iodine diet and 3.5 millicuries per kg. of body weight of I^{131}, there was no gross or microscopic evidence of thyroid tissue. A tracer study using 50 microcuries of I^{131} per rat (Table 3) demonstrated a greater I^{131} excretion for 48 hours following the tracer dose than occurred in untreated animals. The distribution of I^{131} excretion in normal and hypothyroid rats following the tracer dose resembles the results obtained in studies on normal and hypothyroid human subjects (Skanse, 1949).

Survival Time: Table 1 summarizes the survival times of hypothyroid dogs and rats following nephrectomy. The hypothyroid rats survived an average of 19.59 hours longer than the normal controls. This difference is highly significant ($P < 0.01$). The small group of rats fed 6-n-propyl-2-thiouracil for a period of 6 weeks before nephrectomy also showed a significantly prolonged survival time ($P < 0.01$). Propylthiouracil given by stomach tube following nephrectomy did not influence the survival time of 25 rats. The hypothyroid dogs had an average increase in survival of 77.9 hours over their controls. This difference is also highly significant ($P < 0.01$).

Blood Composition Following Nephrectomy: The serum potassium values 72 hours after nephrectomy averaged 1.6 meq/1 (Table 2) higher for the euthyroid group of rats. This difference is of doubtful significance ($P < 0.10$). There was no significant difference in serum non-protein nitro-

[5] Cholesterol values before I^{131} averaged 134.4 mg%, ranging from 90 to 185 mg%. Following thyroidectomy (I^{131}) the average cholesterol value was 317 mg%, ranging from 200 to 410 mg%.

TABLE 2. COMPARISON OF SERUM POTASSIUM, INORGANIC PHOSPHORUS AND NON-PROTEIN NITROGEN BETWEEN NORMAL AND THYROIDECTOMIZED RATS 72 HOURS AFTER NEPHRECTOMY

	No. of determinations	Potassium*		Inorganic phosphorus*		Non-protein nitrogen*	
		meq./1	S.D.	mgm. %	S.D.	mgm. %	S.D.
Control	6	8.1	1.38	26.7	3.35	325.3	55.0
Thyroideztomized (I^{131})	6	6.5	1.13	19.7	3.32	290.5	62.1

* Serum values.

gen values of the two groups ($P < 0.40$). However, the hypothyroid rats had a distinctly lower (7 mg%) serum inorganic phosphorus concentration 72 hours after nephrectomy; this difference between the two groups was highly significant ($P < 0.01$).

The serum non-protein nitrogen rise (Fig. 1) was approximately at the same rate in the normal nephrectomized and thyroidectomized-nephrectomized dogs, but the final concentrations are higher in the hypothyroid animals owing to their longer survival time. The rate of increase in serum potassium (Fig. 1) was approximately the same in the two groups, with a higher final concentration in the hypothyroid dog. The rate of inorganic phosphorus rise (Fig. 1) was more rapid in the normal than in the thyroidectomized dogs, but again, the final concentrations are much higher in the hypothyroid animals.

DISCUSSION

The data presented provide convincing evidence that suppression of thyroid function contributes materially to the survival time of acutely renal insufficient rats and dogs. Understanding the significance of this difference will involve an appreciation of the effects of thyroid hormone or its deficiency on the organism. According to Hoberman (1950), the hypothyroid rat undergoes an increased rate of amino acid catabolism associated with a decreased rate of protein breakdown. Judging by the similarity of the rise in non-protein nitrogen in hypothyroid and normal animals follow-

TABLE 3. COMPARISON OF THE URINARY EXCRETION OF A TRACER DOSE OF I^{131} BETWEEN NORMAL AND THYROIDECTOMIZED RATS

		Per cent excretion of I^{131} in urine			
Treatment	Number of animals	24 hrs.	S.D.	48 hrs.	S.D.
Control	3	54.3	2.56	62.3	1.37
Thyroidectomized (I^{131})	4	56.0	1.38	90.2	3.55

Dose: 50 microcuries I^{131} per rat.

Note the increased urinary excretion of I^{131} in the hypothyroid rats during the second 24 hour period.

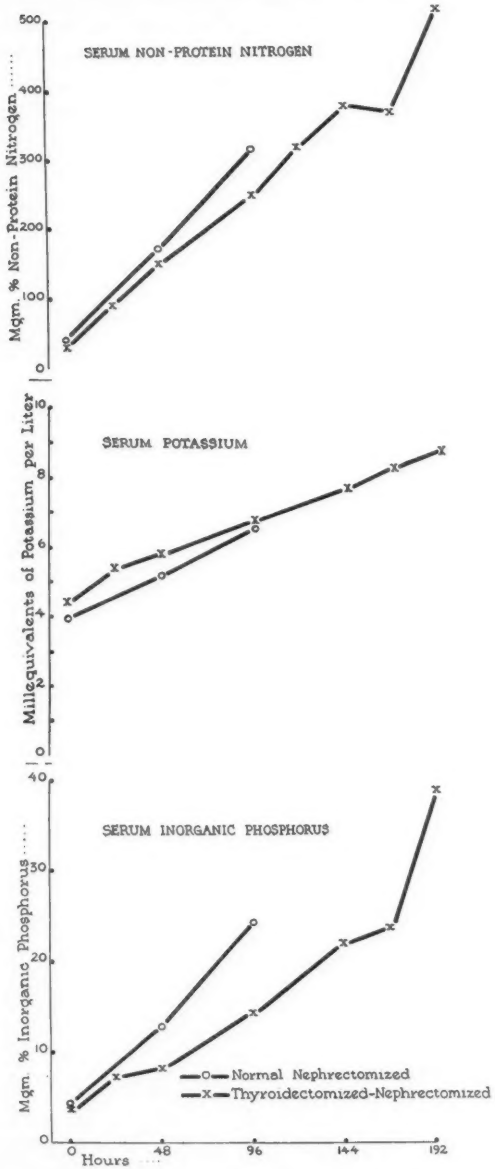

FIG. 1. Changes in blood serum concentration of constituents following nephrectomy in normal and hypothyroid dogs. The number of animals ( ) upon which each average value is based follows: Normal nephrectomized groups: 0 hrs.—(12), 48 hrs.—(12), 96 hrs.—(8); Hypothyroid nephrectomized group: 0 hrs—(9), 24 hrs.—(9), 48 hrs.—(9), 96 hrs.—(9), 144 hrs.—(6), 168 hrs.—(5), 192 hrs.—(5).

ing nephrectomy, the increased catabolism of amino acids in the hypothyroid animal largely offset the reduced protein catabolism during the period of uremia both in dogs and rats. Other workers have reported an increased nitrogen excretion in hypothyroid rats (Persike, 1948; Rupp, Paschkis, and Cantarow, 1949). Cagan, *et al.* (1950) have reported an increase in the amino acid oxidase in the kidney tissue of thyroidectomized rats and believes that the change in enzyme activity produces an increase in amino acid metabolism.

The sites of protein mobilization during starvation may be different in the euthyroid and hypothyroid animal. Adrenal atrophy, shown to occur in the hypothyroid animal (Deane and Greep, 1947) may be a factor in altering the pattern of tissue catabolism. White and Dougherty (1947) reported that there was a predominant loss of lymphoid tissue in hypothyroid mice, whereas in normal fasting mice there was an increased mobilization of carcass protein. According to Addis, Poo and Lew (1936) 62% of the protein lost during a 7-day fast by a normal rat is derived from the carcass. The parallelism in rise of serum non-protein nitrogen and potassium of hypothyroid and normal nephrectomized dogs suggests that the tissues catabolized were similar in the two groups. However, the relatively less potassium than nitrogen mobilized in the hypothyroid rat suggests that there is a qualitative as well as a quantitative difference in the catabolism of hypothyroid and euthyroid rats.

In both species there was a significantly slower rate of serum inorganic phosphorus rise in the hypothyroid group. This difference may be ascribed to a slower rate of breakdown of phosphorus compounds in the hypothyroid animals. *In vitro* studies reported by Askonas (1951) indicate a noncompetitive inhibition of creatine phosphokinase by thyroxine. A reduced rate of nucleic acid catabolism may be a factor in the slower rate of accumulation of inorganic phosphorus in the extracellular fluid of the hypothyroid-nephrectomized animal.

The higher final concentration of serum non-protein nitrogen, potassium, and phosphate in the hypothyroid-nephrectomized dogs indicates a greater total tissue breakdown during their survival period than occurred in the normal-nephrectomized animals. This finding suggests that neither the nitrogenous nor inorganic products derived from protein breakdown were in themselves the limiting factor of survival following nephrectomy.

The effect of protein on survival of uremic animals cannot be attributed to any extracellular electrolyte change, according to Coats and Wellby (1952). Harris, *et al.* (1952) found no changes in the plasma electrolytes sufficient to account for the death of uremic sheep. However, there is a considerable body of evidence that protein catabolism is in some way re-

lated to the tolerance of the animal for renal insufficiency. Also, it must be acknowledged that changes in the extracellular electrolytes may contribute materially to the deterioration of the renal insufficient organism.

SUMMARY

It has been shown that there is a significant increase in the survival time following nephrectomy of hypothyroid dogs and rats as compared to their controls.

Studies of the serum inorganic phosphorus, non-protein nitrogen and potassium indicate that the increased survival time of the hypothyroid-nephrectomized animal is accompanied by a significant reduction in the rate of accumulation of inorganic phosphorus in the serum of these animals.

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GLYCOGENOLYTIC EFFECTS OF EPINEPHRINE IN SKELETAL MUSCLES OF HYPOPHYSECTOMIZED RATS TREATED WITH GLYCOPEXIC HORMONES[1]

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IN A previous study, it was shown that in normal female rats, cortisone acetate inhibited the glycogenolytic action of epinephrine in the rectus femoris and abdominal muscles but not in the diaphragm (Wortman and Leonard, 1953). On the other hand, adrenalectomy increased the glycogenolytic effect of epinephrine in rat skeletal muscle (Collip, Thomson and Toby, 1936) but this could be prevented by treatment with adrenal cortical extracts (Winternitz and Long, 1952). Epinephrine administered intravenously to fed normal and hypophysectomized rats reduced muscle glycogen equally in both instances, but if given subcutaneously, epinephrine was less effective in hypophysectomized rats (Russell and Cori, 1937). It appears that the antagonistic action between glycogenolytic and glycopexic hormones in different skeletal muscles requires further study, particularly since it is usually inferred that skeletal muscles react similarly.

Since muscle glycogen of fasting hypophysectomized rats can be increased or maintained at normal levels by a number of glycopexic hormones (Russell and Wilhelmi, 1950; Leonard, 1953), a study has been made on the glycogenolytic effects of epinephrine in fasting hypophysectomized rats pretreated with growth hormone, cortisone acetate and thyroxine. Special attention has been given to comparative effects in different skeletal muscles.

METHODS

Adult female rats of the Long-Evans strain weighing 200–240 grams were used. They were fed a stock diet of calf meal and water, and in addition, a 10% solution of glucose was given to the hypophysectomized animals.

Two samples of growth hormone preparations were employed but the daily dose of each was approximately equivalent, based on a reference standard determined at the source.[2] The daily dose of preparation K40105R was 0.375 mg. and was equivalent to

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[2] The growth hormone preparations were supplied through the courtesy of Dr. Irby Bunding, Armour Research Laboratory, Chicago, Illinois.

1.05 mg. of preparation K491082P. In one experiment, the daily dose was increased 5 times. The growth hormone was dissolved in saline with a small amount of 0.1 N NaOH added to facilitate solution. Cortisone acetate crystals[3] were ground finely, suspended in water and injected in daily doses of 2 and 5 mg. in different experiments. Crystalline thyroxine was dissolved in a slightly alkaline saline solution and given in daily doses of 150 micrograms. All hormones were given in 1 cc. volumes daily for 3 successive days. Growth hormone was injected in divided doses twice daily intraperitoneally; cortisone and thyroxine were given subcutaneously once daily. Where combinations of the above hormones were given, only the smaller doses were injected.

Treatment with these glycopexic hormones was begun one week after hypophysectomy. The food and sugar solution were removed 24 hours before autopsy. In each experiment consisting of 10–12 rats, half received intraperitoneally, 5 μg. of epinephrine per 100 gm. body weight (weighed after fasting), and the controls, saline. The intraperitoneal method of administration was adopted in order to circumvent the undesirable features of subcutaneous administration (slower absorption in the hypophysectomized rat), and of the intravenous method (necessary manipulation of the rats). After one hour, the rats were anesthetized with sodium pentabarbital (6 mg. per 100 gm. body weight). Five minutes after giving the anesthetic, one rectus femoris, a portion of the abdominal muscles and half the diaphragm were removed, weighed and placed in tubes containing KOH. The tubes were immediately placed in a boiling water bath, and allowed 30 minutes for complete digestion. The details of these procedures and the subsequent glycogen determination by the anthrone method are given in a previous report (Leonard, 1953). The glycogen is reported as mg. of glucose per 100 gm. of muscle, wet weight (averages with the standard error of the mean). Data is presented only on completely hypophysectomized rats.

EXPERIMENTAL

The injection of epinephrine into fasting hypophysectomized rats decreased the glycogen content of the rectus femoris, abdominal and diaphragm muscles significantly but not to the same extent in each muscle (Table 1). Similar treatment with epinephrine had been previously used to induce glycogenolysis in these muscles in fasting intact female rats (Wortman and Leonard, 1953). A comparison of the net loss of glycogen which occurred in the hypophysectomized rats, with that observed previously in intact rats under similar conditions, indicated that less glycogen disappearred from the muscles of the hypophysectomized rats, except in the diaphragm (controls in Tables 1 and 2). However, a comparison of the amount of glycogen lost with respect to the initial levels (per cent loss) for each muscle, indicated there was no essential difference in the glycogenolytic effects of epinephrine between *fasting* intact and hypophysectomized rats. The data of Russell and Cori (1937) showed that the per cent loss of glycogen from the gastrocemius muscle of *fed* normal and hypophysectomized rats, given epinephrine intravenously, is also essentially the same.

[3] Cortisone acetate was obtained through the courtesy of Dr. Preston Perlman, Schering Corporation, Bloomfield, New Jersey.

Cortisone acetate. When hypophysectomized rats, pretreated with cortisone acetate (2 mg. doses), were injected with epinephrine, a significant decrease in the level of glycogen occurred but differing in amount in each muscle (Table 1).

The net loss of glycogen in the cortisone-treated as compared to the control rats was the same in the rectus femoris but greater in the abdominal and diaphragm muscles. A comparison of the per cent loss (in relation to the initial glycogen levels) showed that epinephrine decreased the glycogen by 16% in the leg muscles of the cortisone-treated rats as compared to 28% in the controls. No difference was observed in the per cent loss in the abdominal muscles but it was slightly greater in the diaphragm (Table 1).

TABLE 1. GLYCOGENOLYTIC EFFECTS OF EPINEPHRINE IN HYPOPHYSECTOMIZED RATS TREATED WITH GLYCOPEXIC HORMONES

Treatment[1]	No. of rats	Glycogen (mg./100 gm. muscle)			
		Control	Epineph.	Net loss	% loss
		(*Rectus femoris muscle*)			
Control	22	213 ± 14	154 ± 10	59	28
Cortisone ac. (s.d.)	22	390 ± 12	326 ± 21	64	16
Cortisone ac. (l.d.)	23	490 ± 31	357 ± 35	133	27
Growth h. (s.d.)	24	337 ± 18	286 ± 16	51	15
Growth h. (l.d.)	24	398 ± 12	363 ± 16	35[2]	9
Thyroxine	9	326 ± 28	210 ± 12	116	36
Growth h. + cortisone	12	678 ± 52	493 ± 31	185	27
Growth h. + thyroxine	12	456 ± 19	404 ± 9	52	11
Growth h. + cortis. + thyroxine	9	518 ± 17	272 ± 23	246	48
		(*Abdominal muscle*)			
Control	22	276 ± 11	140 ± 9	136	49
Cortisone ac. (s.d.)	20	468 ± 13	262 ± 16	206	44
Cortisone ac. (l.d.)	22	530 ± 29	264 ± 31	266	50
Growth h. (s.d.)	24	446 ± 21	264 ± 10	182	41
Growth h. (l.d.)	24	447 ± 17	334 ± 15	113	25
Thyroxine	9	341 ± 46	131 ± 13	210	62
Growth h. + cortisone	12	751 ± 47	429 ± 45	322	43
Growth h. + thyroxine	12	434 ± 30	246 ± 18	188	43
Growth h. + cortis. + thyroxine	8	555 ± 17	173 ± 16	382	69
		(*Diaphragm*)			
Control	21	211 ± 5	130 ± 6	81	38
Cortisone ac. (s.d.)	20	337 ± 19	180 ± 17	157	47
Cortisone ac. (l.d.)	22	410 ± 18	151 ± 13	259	63
Growth h. (s.d.)	24	256 ± 13	155 ± 6	101	40
Growth h. (l.d.)	12	261 ± 9	101 ± 16	160	61
Thyroxine	9	249 ± 25	94 ± 11	155	62
Growth. h. + cortisone	12	416 ± 25	173 ± 27	243	58
Growth h. + thyroxine	12	249 ± 15	117 ± 8	132	53
Growth h. + cortis. + thyroxine	9	409 ± 32	184 ± 23	225	55

[1] The (s.d.) refers to "small doses"; (l.d. refers to "large doses." Where combinations are used "small doses" only were employed.

[2] This difference not significant. All other differences significant. ($P = .05$ or less).

The limited inhibition of the glycogenolytic effects of epinephrine in the cortisone-treated rats suggested that possibly inadequate amounts of this steroid were employed. The experiment was repeated but the daily dose was increased to 5 mg. per day. The result was that more glycogen was deposited in all the muscles than at the lower dose level. When epinephrine was injected, a greater net loss of glycogen was observed in all the muscles than in the preceding experiment (Table 1). Moreover, the loss of glycogen in the leg muscle was 27% which was the same as that found in the untreated controls. The loss of glycogen in the abdominal muscles was similar to that of the untreated controls whereas in the diaphragm, this increased to 63% as compared to 38% in the respective control.

Growth Hormone. In view of the weak inhibitory action of cortisone acetate on glycogenolysis in the hypophysectomized rat, growth hormone was employed to determine if it would antagonize the effects of epinephrine. Although treatment with growth hormone raised the glycogen level in the rectus femoris considerably above that found in the hypophysectomized controls, the net loss in glycogen, after epinephrine, was similar to that induced in the controls. However, this loss amounted to 15% of the initial level as compared to 28% in the controls. The abdominal and diaphragm muscles of the treated rats responded by a net loss in glycogen greater than that noted in the respective controls but on a per cent basis, the results were very similar.

By increasing the doses of growth hormone 5 fold, however, the glycogenolytic action of epinephrine was inhibited to a greater extent. The slight net loss of glycogen in the leg muscles of the treated rats was not statistically significant. Furthermore, the net loss and per cent loss of glycogen in the abdominal muscle were also less than that observed in the controls, but in the diaphragm, glycogenolysis was greatly increased.

In view of these results, the question arose whether growth hormone would inhibit the effect of epinephrine in intact rats. A group of normal female rats were injected with the small doses of growth hormone and half received epinephrine. The glycogen content in all three muscles of the treated rats was elevated above that of the untreated controls. However, there was no inhibition of glycogenolysis in any of the muscles (Table 2). A greater net loss of glycogen occurred in the growth hormone-treated rats compared to the untreated controls, particularly in the leg and abdominal muscles, but the per cent loss was approximately the same.

Thyroxine. Although optimum amounts of thyroxine are reported to elevate the carbohydrate levels in hypophysectomized animals (Soskin and Levine, 1952), the specific effects on muscle glycogen in hypophysectomized rats first had to be made under conditions set forth in this study. When thyroxine was given in 150 μg. doses to 7 hyophysectomized rats, the aver-

TABLE 2. GLYCOGENOLYSIS INDUCED BY EPINEPHRINE IN MUSCLES OF NORMAL RATS GIVEN GROWTH HORMONE

Treatment	No. of rats	Glycogen (mg. per 100 gm./ muscle)			
		Control	Epineph.	Net loss[1]	% loss
		(*Rectus femoris*)			
Growth h.	10	547 ± 18	378 ± 36	159	30
Controls[2]	22	349 ± 17	261 ± 20	88	25
		(*Abdominal m.*)			
Growth h.	10	605 ± 19	357 ± 20	248	41
Controls[2]	22	371 ± 22	178 ± 22	193	52
		(*Diaphragm*)			
Growth h.	10	294 ± 22	203 ± 13	91	31
Controls[2]	22	232 ± 8	152 ± 15	80	35

[1] Differences are all significant ($P < .01$).
[2] Data from Wortman and Leonard (1953).

age glycogen content of the rectus femoris was 306 ± 16 mg. per 100 gm. muscle as compared to 205 ± 12 mg. for 7 controls. Thyroxine did not elevate the glycogen level in the other two muscles in this experiment.

A small group of hypophysectomized rats were given thyroxine followed by epineprhine to determine the effect on glycogenolysis. The results showed that in all muscles, the net loss and per cent loss of glycogen was greater than that which occurred in the untreated controls (Table 1).

Combinations of glycopexic hormones. Considering the possibility that concurrent administration of two or more glycopexic hormones also might alter the effect of epinephrine, the following experiments were performed. When injections of cortisone acetate and growth hormone were given concurrently at the low dose levels, glycogenolysis was not inhibited. The muscle glycogen in the controls receiving the glycopexic hormones were markedly increased, but epinephrine removed more glycogen from each muscle. The per cent loss of glycogen in the leg and abdominal muscles was similar to that observed in the untreated hypophysectomized controls but in the diaphragm it was considerably higher.

The combination of growth hormone plus thyroxine administered to hypophysectomized rats increased muscle glycogen above that produced by growth hormone alone in the leg muscle only. The effect of epinephrine in this instance was similar to that observed in rats pretreated with growth hormone alone. Finally, a group of rats were injected with the three glycopexic hormones concurrently and half of the group received epinephrine. Glycogen deposition in all three muscles was greatly increased. However, the glycogenolysis which followed epinephrine treatment was greater than in any of the other experiments, not only in the per cent loss of glycogen but also in the net loss. This was particularly obvious in the leg and abdominal muscles (Table 1).

DISCUSSION

Growth hormone in sufficient amounts was very effective in inhibiting the glycogenolytic action of epinephrine in skeletal muscles of hypophysectomized rats in contrast to the effects of cortisone acetate or thyroxine. Both the net loss and per cent loss of muscle glycogen was significantly less than that observed in the hyophysectomized controls injected with the same quantity of epinephrine. The inhibition of glycogenolysis was most clearly demonstrated in the rectus femoris, less in the abdominal muscles but not at all in the diaphragm. The inhibitory effect of small doses of growth hormone was limited and confined to the leg muscle only. Further evidence of the inhibitory effects of growth hormone was observed when growth hormone and thyroxine were injected concurrently. Thyroxine increased glycogenolysis following epinephrine treatment, but in the presence of growth hormone, the inhibitory action of the growth hormone predominated. In view of these results, it was surprising that growth hormone failed to antagonize the action of epinephrine in intact female rats.

Cortisone acetate, in 2 mg. doses, inhibited the effect of epinephrine only in the leg muscle, much the same as did small doses of growth hormone. With larger doses of cortisone acetate, the inhibitory effect was reversed. Not only was more glycogen lost from the leg muscle but the per cent loss was similar to that obtained in the untreated controls. Large doses of cortisone are reported to be detrimental to the structural integrity of skeletal muscles (Ellis, 1952). Possibly some changes may have occurred in the muscles of the cortisone-treated hypophysectomized rats which rendered them more sensitive to epinephrine. In contrast to these findings, cortisone acetate readily inhibited glycogenolysis in both the leg and abdominal muscles of the intact female rat (Wortman and Leonard, 1953).

Although thyroxine increased glycogen storage in the leg muscle, its influence in potentiating the glycogenolytic effect of epinephrine predominated. Collip, *et al.* (1936), reported that thyroxine increased muscle glycogenolysis in fed hypophysectomized rats injected subcutaneously with epinephrine.

The concurrent treatment with growth hormone and cortisone acetate appeared to cancel the inhibition of glycogenolysis which was apparent when these hormones were given separately (in the leg muscle only). The per cent loss of glycogen was the same as in the control group. When all three glycopexic hormones were given concurrently, the glycogenolytic action of epinephrine was strikingly increased.

Throughout these experiments, there was no evidence of inhibition of glycogenolysis in the diaphragm muscle of the hypophysectomized rats, regardless of the treatment. Previously, it was shown that cortisone acetate

could neither elevate glycogen nor inhibit glycogenolysis in the diaphragm of intact rats (Wortman and Leonard, 1953). These results and those of Illingworth and Russell (1951) point to the unique behavior of the diaphragm muscle as contrasted to other skeletal muscle, in response to hormonal regulation of glycogen storage.

Some criticism might be directed against the use of fasting animals in these experiments, especially when comparisons are made between the effects of epinephrine in fasted normal and hypophysectomized animals. Russell and Cori (1937) cautioned against making such comparisons because of the inability of the hypophysectomized animal, deprived of food for short periods, to maintain normal levels of muscle glycogen. However, if one compares the per cent loss of glycogen in fasted normal and hypophysectomized rats following the same treatment with epinephrine, the results are similar in each of the 3 muscles (controls in Tables 1 and 2). Although it is possible to maintain the levels of muscle glycogen with hormone treatment in fasted hypophysectomized rats as high or higher than that obtained in fed hypophysectomized rats, the glycogenolytic action of epinephrine is modified according to the glycopexic hormone employed and the skeletal muscle designated.

SUMMARY

The glycogenolytic effect of epinephrine on muscle glycogen has been studied in fasting hypophysectomized female rats, pretreated with growth hormone, cortisone acetate, thyroxine and several combinations of these glycopexic hormones. Comparative effects were noted on the rectus femoris, abdominal and diaphragm muscles. Growth hormone was most effective in inhibiting glycogenolysis in hypophysectomized rats but was unable to do so in normal rats. Cortisone acetate slightly inhibited glycogenolysis in the leg muscle when given in low doses, but larger doses reversed this effect. Thyroxine alone, and the combined action of the three glycopexic hormones potentiated the glycogenolytic effect of epinephrine. Inhibition of glycogenolysis could not be demonstrated in the diaphragm, but instead, the effect of epinephrine was increased by all treatments.

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PHOSPHORUS METABOLISM OF THE ADRENAL GLAND: EFFECT OF HYPOPHYSECTOMY AND ADMINISTRATION OF ACTH ON THE INCORPORATION OF RADIOACTIVE PHOSPHATE INTO PHOSPHOLIPID[1]

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GEMZELL and Samuels (1950) investigated the incorporation of inorganic phosphate labeled with P^{32} into the inorganic phosphate fraction of a number of organs of normal and hypophysectomized rats. They found that hypophysectomy caused a decrease in the specific activity of the inorganic phosphate of the adrenal gland relative to that of the inorganic phosphate of the plasma. A normal rate of incorporation of P^{32} into the inorganic phosphate of the adrenal was largely restored by a single injection of ACTH. Reiss and Halkerston (1950) made similar observations for the total acid-soluble phosphorus fraction of the adrenal gland. These findings were, in the main, confirmed by Riedel, Logan, DeLuca and Rossiter (1954). Evidence was presented by Riedel and Rossiter (1954) for the suggestion of Gemzell and Samuels (1950) that in the adrenal gland of hypophysectomized rats there is a defect in the passage of inorganic P^{32} from the extracellular to the intracellular portion of the gland.

In the experiments described in this paper, the lipid phosphorus of the adrenal has been studied. It is shown that both the concentration of lipid phosphorus, and the incorporation of P^{32} into the lipid phosphorus 16 hours after the administration of P^{32}, are decreased in the adrenals of hypophysectomized rats. A single injection of ACTH causes the concentration of lipid phosphorus to return towards normal and largely restores the normal rate of incorporation of P^{32} into the lipid phosphorus.

METHODS

Animals

The animals were male rats of the Sprague-Dawley strain. Hypophysectomized rats were supplied by the Hormone Assay Laboratories, Inc., Chicago. Macroscopic and microscopic examination of the pituitary region showed no evidence of pituitary tissue

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remaining. The adrenal glands displayed the typical atrophy associated with hypophysectomy. The animals were fasted 12–14 hours before killing. Control animals were carried along with each group of test animals.

ACTH Preparations

Two preparations of ACTH were used. ACTH A was obtained from Armour and Company, Chicago (Batch #128-105). It was a buff-colored powder that formed a slightly turbid suspension in isotonic saline. ACTH C was supplied by the Connaught Medical Research Laboratories, Toronto. It was a white powder, completely soluble in isotonic saline. Both preparations had been standardized in terms of the international standard. No significant difference was noted in the effectiveness with which the two preparations promoted the incorporation of P^{32} into the adrenal lipids.

Test animals received a single intraperitoneal injection of ACTH, 4 mg. per 100 gm. body weight, in isotonic saline. Control animals received an equivalent volume of isotonic saline. Four hours later all the animals received 200 μc. P^{32}*, regardless of body weight, by intraperitoneal injection. The volume of each injection was never more than 0.5 cc.

The animals were killed 16 hours after the P^{32} injection, i.e. 20 hours after the injection of ACTH or saline. Under nembutal anaesthesia, a midline incision was made in the abdomen at the height of the diaphragm. A sample of blood was withdrawn from the inferior vena cava into a 2 cc. syringe containing a small quantity of heparin. The blood was transferred, with care to prevent hemolysis, to a centrifuge tube that was kept in an ice-bath. The adrenal glands were removed as rapidly as possible, blotted to remove excess blood, trimmed carefully to remove adhering adipose and connective tissue, and rapidly frozen by plunging into liquid nitrogen. The blood sample was then centrifuged in the cold room.

Analytical and Counting Methods

The two adrenals were pooled and weighed in the frozen state. They were homogenized in ice-cold 10% trichloroacetic acid using a homogenizer of the Potter-Elvehjem type. The concentration and the specific activity of the inorganic P of the trichloroacetic acid extracts of the plasma and adrenals were then determined by the method of Ernster, Zetterström and Lindberg (1950). The tissue residue was washed five times with 10% trichloroacetic acid in the cold to remove the last traces of acid-soluble radioactivity. The residue was then extracted successively with ethanol (2 portions), ethanol-ether 3:1 (3 portions heated to boiling) and ether. A portion of the combined extract was ashed with 60% perchloric acid by the method of King (1932) and the concentration and the specific activity determined as described for the inorganic P.

In the method of Ernster *et al.* (1950) both the concentration and the radioactivity of the inorganic P are determined on the same sample. The optical density of the blue color complex was read with a Beckman Model B Spectrophotometer at 730 mμ and the radioactivity due to the P^{32} in the samples was determined on the same colored solution using an M-6 liquid counter (20th Century Electronics). The counting rate was corrected for background and decay. The specific activities for each animal were adjusted to a standard injection of 200 μc. per 200 gm. body weight, based on a simulated P^{32} standard (Tracerlab. Inc., Boston).

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* Supplied in the form of $H_3P^{32}O_4$ by Eldorado Mining and Refining (1944) Limited.

TABLE 1. EFFECT OF HYPOPHYSECTOMY ON THE COMBINED ADRENAL WEIGHT AND THE CONCENTRATION OF LIPID PHOSPHORUS IN THE ADRENAL GLAND OF THE RAT. MEAN ±S.E. MEAN

Group	No. animals	Combined adrenal weight (mg.)	Lipid P (mg./100 gm.)	Total lipid P (μg./pr. adrenals)
Hypophysectomized 14 days	12	13.7±0.8	89.0±2.6	12.2
Hypophysectomized 6 days	17	17.0±2.6	93.8±1.6	16.0
Hypophysectomized 4 days	4	23.4±5.0	107.3±5.7	25.1
Hypophysectomized 2 days	23	24.0±4.5	117.5±2.1	28.2
Normal	47	34.9±6.3	139.5±1.7	47.4

RESULTS

Concentration of Lipid Phosphorus

Table 1 shows the usual fall in the combined weight of the adrenal glands observed after hypophysectomy. In addition to a fall in the adrenal weight, hypophysectomy also caused a decrease in the concentration of lipid P. It is thus evident that in hypophysectomized animals a considerable quantity of phospholipid leaves the gland. This is demonstrated by the changes in total lipid P (per pair of adrenals) shown in the last column of Table 1.

Twenty hours after a single intraperitoneal injection of ACTH into animals hypophysectomized 2 days previously, the concentration of lipid P in the adrenal was significantly increased (Table 2). At 6 hours after the injection there was an increase, but this was not significant statistically. Each of the preparations of ACTH was effective.

TABLE 2. EFFECT OF ADMINISTRATION OF ACTH ON THE CONCENTRATION OF LIPID PHOSPHORUS IN THE ADRENAL GLAND OF THE HYPOPHYSECTOMIZED RAT

Time after hypophysectomy, 2 days. Mean±S.E. Mean

Group	No. animals	Time after ACTH (hr.)	Lipid P (mg./100 gm.)	*P*	
Hypophysectomized	23	—	117.5±2.1	>0.3	>0.3
Hypophysectomized+ACTH A	21	6	120.5±2.7		
Hypophysectomized+ACTH C	14	6	120.6±3.0		
Normal	47	—	139.5±1.7		
Hypophysectomized	18	—	114±3.5	<0.01	<0.01
Hypophysectomized+ACTH A	19	20	128±3.1		
Hypophysectomized+ACTH C	20	20	125±2.3		
Normal	8	—	139±5.8		

TABLE 3. EFFECT OF HYPOPHYSECTOMY AND THE ADMINISTATION OF ACTH ON THE SPECIFIC ACTIVITIES OF THE INORGANIC P OF THE PLASMA AND THE INORGANIC PHOSPHORUS AND LIPID PHOSPHORUS OF THE ADRENAL GLAND OF THE RAT

Time after ACTH, 20 hr. Time after P^{32}, 16 hr. Mean ±S.E. mean

Group	No. animals	Plasma Inorg. P (cts./min. /μg.P)	Adrenal inorg. P (cts./min. /μg.P)	Adrenal lipid P (cts./min. /μg.P)
Hypophysectomized 2 days	18	130 ±7.2	103± 6.9	27.4± 5.0
Hypophysectomized+ACTH A	19	117 ±8.8	122 ±10.7	44.4 ±13.0
Hypophysectomized+ACTH C	20	111 ±9.1	112± 6.9	39.3 ±10.7
Normal	8	134 ±7.3	121± 6.3	58.1± 5.0
Normal+ACTH A	8	133 ±3.0	131± 5.1	61.0± 4.2
Normal ±ACTH C	8	129 ±6.6	130± 8.7	67.7± 7.2

Specific Activity of Lipid Phosphorus

The specific activities of the inorganic P of the plasma, the inorganic P of the adrenal, and the lipid P of the adrenal are shown in Table 3. For rats hypophysectomized 2 days previously, and at 16 hours after the administration of P^{32}, there was little difference between the specific activities of the plasma inorganic P of normal and hypophysectomized animals. The specific activity of the inorganic P of the adrenal was decreased slightly in the hypophysectomized animals, but the decrease was not nearly as great as that reported by Riedel *et al.* (1954) for animals studied 2 hours after the P^{32} injection. The specific activity of the lipid P was much less in the hypophysectomized animals. When the specific activity of the lipid P was calculated relative to that of the inorganic P of the plasma, the value for the hypophysectomized animals was considerably less than that for the normal animals (Table 4). The administration of either preparation of ACTH caused a significant increase in the hypophysectomized animals, but was without a significant effect in the normal animals.

Since it is known that in hypophysectomized animals there is a decrease

TABLE 4. EFFECT OF HYPOPHYSECTOMY AND THE ADMINISTRATION OF ACTH ON THE SPECIFIC ACTIVITY OF THE LIPID PHOSPHORUS OF THE ADRENAL GLAND OF THE RAT RELATIVE TO THE SPECIFIC ACTIVITY OF THE INORGANIC PHOSPHORUS OF (A) THE PLASMA AND (B) THE ADRENAL GLAND

Time after ACTH, 20 hr. Time after P^{32}, 16 hr. Mean ±S.E. mean

Group	No. animals	Rel. sp. activ. (plasma inorg. P) $\times 10^2$	P		Rel. sp. Activ. (adrenal inorg. P) $\times 10^2$	P	
Hypophysectomized 2 days	18	21.1±1.1	<0.001	<0.001	25.6±1.2	<0.001	<0.001
Hypophysectomized+ACTH A	19	39.4±4.1			37.0±2.2		
Hypophysectomized+ACTH C	20	34.5±2.8			34.2±1.3		
Normal	7	46.4±6.2	>0.3	>0.7	48.4±3.9	>0.6	>0.5
Normal+ACTH A	8	46.0±3.1			46.3±1.4		
Normal−ACTH C	8	52.2±4.4			51.4±2.7		

in the specific activity of the inorganic P of the adrenal gland relative to that of the inorganic P of the plasma, greatest during the early time intervals after the injection of the P^{32} (Riedel and Rossiter, 1954), the figures for the specific activity of the lipid P were calculated relative to the specific activity of the inorganic P of the adrenal. The last column of Table 4 shows that when the results were expressed in this manner, the relative specific activity (adrenal inorganic P) of the lipid P of the adrenals of the hypophysectomized animals still remained less than the corresponding figure for the control animals. The administration of either preparation of ACTH again caused a significant recovery in hypophysectomized animals, whereas in normal animals ACTH was without a significant effect.

The Change in Phospholipid Metabolism after Hypophysectomy

The phospholipid metabolism of rats hypophysectomized 14 days previously was investigated in more detail. Groups of control and hypophysectomized rats of similar body weight were studied at intervals of 2, 4, 8, 16 or 24 hours after the injection of the P^{32}.

Table 5 shows the specific activities of the inorganic P of both the plasma and adrenal gland at different time intervals after the injection of the P^{32}. The specific activity of the inorganic P of the plasma of the hypophysectomized animals was considerably greater than that of the normal animals at each of the time intervals. The values for the hypophysectomized rats two hours after the injection were considerably greater than those reported by Riedel *et al.* (1954) for rats hypophysectomized 2 days previ-

TABLE 5. SPECIFIC ACTIVITY OF INORGANIC PHOSPHORUS OF PLASMA AND ADRENAL GLANDS OF NORMAL AND HYPOPHYSECTOMIZED RATS AFTER AN INTRAPERITONEAL INJECTION OF P^{32}

Figures in parentheses indicate the number of animals in each group

Time after P^{32} (hr.)	Plasma inorg. P(A) (cts./min. μg.P)		Adrenal inorg. P (B) (cts./min./μg.P)		Adrenal inorg. P relative to plasma inorg. P (B/A×100)	
	Normal	Hypothy-sectomized	Normal	Hypophy-sectomized	Normal	Hypophy-sectomized
2	458 (59)	851 (11)	343 (56)	352 (12)	76.0±3.2	41.3±1.6
4	215 (10)	574 (5)	270 (10)	313 (5)	132±9.6	54.9±2.7
8	149 (10)	331 (5)	163 (10)	222 (5)	114±6.2	69.3±10.6
16	101.5 (10)	158 (6)	122 (9)	119 (6)	119±7.2	74.9±5.2
24	76.1 (11)	125 (5)	96.4 (11)	97.0 (5)	127±4.8	76.9±6.3

ously, but they were only slightly greater than those reported for rats studied 6 days after hypophysectomy.

As was found previously for rats studied 6 days after hyophysectomy, there was little difference between the specific activities of the inorganic P of the adrenal of normal and hypophysectomized rats (Riedel *et al.*, 1954). In both groups the specific activity of the inorganic P of the plasma and the specific activity of the inorganic P of the adrenal decreased with time after the P^{32} injection, but that of the plasma fell faster than that of the adrenal, so that the specific activity of the inorganic P of the adrenal relative to that of the inorganic P of the plasma increased with time.

The last two columns of Table 5 shows that there was a considerable reduction in the specific activity of the inorganic P of the adrenals relative to that of the inorganic P of the plasma in the hypophysectomized rats. This difference, previously reported by Gemzell and Samuels (1950) and Riedel *et al.* (1954), became less with increase in time after the injection of the P^{32}. Reasons are given by Riedel and Rossiter (1954) for supposing that in hypophysectomized rats this decrease in the relative specific activity of the inorganic P of the adrenal is due to a decrease in the rate at which inorganic P^{32} passes through the membrane of the adrenal cell.

In addition to the decrease in the relative specific activity of the inorganic P in the adrenal gland of hypophysectomized rats, there was also a decrease in the specific activity of the lipid P of the adrenal gland and in the specific activity of the lipid P relative either to that of the inorganic P of the plasma or to that of the inorganic P of the adrenal itself at all time intervals after the injection of the P^{32} (Table 6). This confirmed, for animals hypophysectomized 14 days previously, the results reported in Table 4 for animals studied 2 days after the removal of the pituitary.

TABLE 6. SPECIFIC ACTIVITY OF LIPID PROPHORUS OF ADRENAL GLANDS OF NORMAL AND HYPOPHYSECTOMIZED RATS AFTER AN INTRAPERITONEAL INJECTION OF P^{32}

Figures in parentheses indicate the number of animals in each group

Time after P^{32} (hr.)	Lipid P (C) (cts./min./μg. P)		Lipid P relative to plasma inorg. P (C/A×100)		Lipid P relative to adrenal inorg. P (C/A×100)	
	Normal	Hypophysectomized	Normal	Hypophysectomized	Normal	Hypophysectomized
4	21.3 (10)	16.4 (5)	10.6±2.1	2.9±0.2	7.9	5.2
8	37.8 (10)	29.5 (5)	25.6±2.1	9.2±1.7	23.1	13.2
16	64.1 (7)	38.7 (5)	65.5±3.1	26.4±3.4	52.6	32.7
24	66.3 (11)	55.6 (5)	87.2±5.3	44.8±4.4	68.9	57.2

Rate of Formation of Phospholipid

If it is assumed that phospholipid is formed in the adrenal gland from inorganic P, or from some compound in rapid equilibrium with inorganic P, with no rate-limiting reaction intervening, the percentage renewal of the lipid P of the adrenal is given by the percentage ratio of the specific activity of the lipid P to the mean specific activity of the intracellular inorganic P during the time interval of the experiment. This time interval must be so chosen that the renewal of phospholipid already labeled is sufficiently small to be ignored. The advantages and limitations of this method of determining rates of renewal have been discussed by several workers (Chaikoff, 1942; Hevesy, 1947; Chaikoff and Zilversmit, 1948; Hevesy, 1948).

In evaluating the accompanying calculations, it must be remembered that the phospholipid of the adrenal, like that of most tissues, is a mixture of a number of chemical entities, which quite likely are renewed at different rates. Concerning the immediate P-containing precursor of phospholipids, little is known. However, Findlay (1954) has shown that inorganic P^{32} is incorporated *in vitro* into the phospholipids of the adrenal gland. This incorporation depends upon the aerobic metabolism of the tissue.

The inorganic P of the adrenal, like that of any other tissue, is partly extracellular (plasma and interstitial fluid) and partly intracellular. After an injection of inorganic P labeled with P^{32}, the isotope rapidly equilibrates with the inorganic P of the extracellular space (Hevesy, 1939; Hahn and Hevesy, 1941). However, the passage of the isotope through the cell membrane into the cells is much slower, the rate depending upon the nature of the tissue studied (for reviews, see Hevesy, 1947, 1948). For the adrenal gland of normal rats, isotope equilibrium between the inorganic P of the extracellular and intracellular fluids is reached in 3 hours, but in hypophysectomized animals, equilibrium is not reached by the end of 24 hours (Riedel and Rossiter, 1954). For the longer time intervals after the injection, therefore, the specific activity of the inorganic P of the adrenal gland will give a good estimate of the specific activity of the intracellular inorganic P for normal animals. For hypophysectomized animals, and for the normal animals 2 hours after the injection of the isotope, the specific activity of the inorganic P of the adrenal must be corrected for the higher specific activity of the inorganic P in the extracellular fluid.

Table 7 shows the calculated values for the intracellular inorganic P of the adrenal of normal and hypophysectomized rats at each of the time intervals studied. The figures were calculated from the specific activity data for the plasma and adrenal inorganic P reported in Table 1 and the concentration data for plasma and adrenal inorganic P for normal and hypophysectomized animals reported by Riedel *et al.* (1954). In the cal-

TABLE 7. CALCULATED SPECIFIC ACTIVITY OF INORGANIC PHOSPHORUS OF INTRACELLULAR FLUID OF ADRENAL GLANDS OF NORMAL AND HYPOPHYSECTOMIZED RATS

Time after P^{32} (hr.)	Intracellular inorganic P (cts./min./μg. P)	
	Normal	Hypophysectomized
2	326	303
4	277	288
8	165	213
16	124	115
24	99	94

culations it is assumed (a) that the volume of the extracellular fluid compartment of the adrenal gland of the rat is 36%, the value obtained by Perlman, Morton and Chaikoff (1941) using the radioactive bromide method, and (b) that the specific activity and the concentration of the inorganic P in the extracellular portion of the gland is the same as that of the plasma (Hevesy, 1948).

Table 8 shows the percentage ratio of the specific activity of the lipid P to that of the mean specific activity of the intracellular inorganic P for normal and hypophysectomized animals. At each time interval the percentage ratio was less for the hypophysectomized animals. In the last two columns of Table 8 the figures have been expressed as the percentage of the lipid present that is renewed per hour up to each of the times shown. For each time the figure was less for the hypophysectomized animals, the mean value of 1.3% per hour being significantly ($P < 0.01$) less than that of 1.7% per hour for the normal animals.

DISCUSSION

Using the factor of 25 to convert lipid P to phospholipid, the concentration of phospholipid in the adrenal of the normal rat was found to be 3.5%

TABLES 8. RENEWAL* OF LIPID PHOSPHORUS IN ADRENAL GLANDS OF NORMAL AND HYPOPHYSECTOMIZED RATS

Time after P^{32} (hr.)	Ratio (S.A. lipid P/Mean S.A. intracell. inorg. P) × 100, i.e. lipid P renewed at end of time interval (percentage of lipid P present)		Lipid P renewed per hr. during time interval (percentage) of lipid P present)	
	Normal	Hypophysectomized	Normal	Hypophysectomized
4	6.5	5.4	1.6	1.4
8	13.8	11.6	1.7	1.4
16	30.6	17.5	1.9	1.1
24	37.5	30.5	1.6	1.3
			$\overline{1.7}$ (±0.07)	$\overline{1.3}$ (±0.07)

Mean (±S.E. Mean)

* Renewal is used subject to the qualifications mentioned in text.

wet weight. This can be compared with the values of 3.5–3.6% reported by Randall and Graubard (1940) and MacLachlan, Hodge and Whitehead (1941) for rabbit adrenal and 4.4–5.7% reported by Oleson and Bloor (1941) and Knouff, Brown and Schneider (1941) for guinea pig adrenal. The concentration of phospholipid in the rat adrenal is of the same order as the concentration of cholesterol, but the changes that occur after hypophysectomy or after the administration of ACTH are in the opposite direction to those observed for cholesterol (summarized by Sayers, Sayers, Fry, White and Long, 1944).

It is of interest to note that in general the amount of lipid demonstrable by histochemical means has been reported to parallel the concentration of cholesterol (Sayers *et al.*, 1944). The histologically demonstrable lipid bears little relation to the concentration of phospholipid, despite the fact that both phospholipid and cholesterol are normally present in the adrenal in similar concentrations. One point that merits further study, especially in relation to the specificity of histochemical tests for lipids, is the report of Rennels (1952) that the injection of ACTH causes a reduction in the amount of material demonstrable by the method of Baker (1946), a method said to be specific for phospholipid. This finding is at variance with the results reported here.

The specific activity figures make it clear that there is a decrease in the incorporation of P^{32} into the phospholipid of the adrenals of hypophysectomized rats. This decrease is prevented by a single intraperitoneal injection of ACTH.

A reduction in the rate at which inorganic P passes through the membrane of the cells of the adrenal gland in hypophysectomized animals was postulated by Riedel and Rossiter (1954). At the outset it seemed reasonable to suppose, therefore, that the decrease in the incorporation of P^{32} into the lipid P might be secondary to this change in phosphorus metabolism at the cell membrane. However, from the results reported here the conclusion seems inescapable that in the adrenal glands of hypophysectomized rats there is also a decrease in the formation of lipid P from intracellular inorganic P. The reduced incorporation of P^{32} into the lipid P can thus be accounted for only in part by the reduced ability of the inorganic P^{32} to penetrate into the adrenal cell.

To state that the reduced phospholipid formation is not secondary to the reduction in the passage of inorganic P^{32} through the cell membrane does not mean that these two effects may not have a similar origin. It is possible that both changes do no more than reflect a marked decrease in the total energy metabolism of the adrenal cell brought about by hypophysectomy. Such a change in energy metabolism might be due to a direct impairment of one or more of the metabolic reactions involved in energy production, or

it might be secondary to the impairment of some reaction involved in the utilization of metabolic energy in the adrenal, e.g. some phase of sterol metabolism.

If the decrease in the rate of passage of P^{32} across the cell membrane is due to a decrease in the energy metabolism of the adrenal cell, it follows that normal metabolism within the cell is necessary for the establishment of normal isotope equilibrium between the inorganic P of the interstitial fluid and that of the intracellular fluid. In this regard it is of interest to note that for muscle cells (Sacks and Altshuler, 1942; Sacks, 1948), yeast cells (Kamen and Spiegelman, 1948), and red blood cells (Gourley, 1952), there is some evidence for the view that, before the inorganic P^{32} of the extracellular fluid can enter the cell, it must first be incorporated into organic phosphorus compounds at the cell membrane. Subsequently these compounds may be broken down to yield inorganic P within the cell.

In favour of the suggestion that both the reduced phospholipid formation and the reduced passage of inorganic P^{32} through the cell membrane are due to a decrease in the metabolism of the cell, is the observation that both effects are rapidly reversed by a single injection of ACTH. The stimulating effect of ACTH on the over-all metabolism of the adrenal is well known. The addition of ACTH *in vitro* to respiring slices of adrenal tissue causes an increase in the oxygen consumption (Carpenter, MacLeod and Reiss, 1946; Tepperman, 1950; Saffran, Bayliss and Webb, 1951), a decrease in the concentration of ascorbic acid (Tepperman, 1950; Saffran, *et al.* 1951), and an increase in the production of corticoids (Saffran, Grad and Bayliss, 1952).

SUMMARY AND CONCLUSIONS

The concentration of lipid phosphorus was decreased in the adrenal glands of hypophysectomized rats, the extent of the reduction depending upon the time after the removal of the pituitary. In rats hypophysectomized 2 days previously a single injection of ACTH caused a significant increase within 20 hours.

The specific activity of the lipid phosphorus of the adrenal glands of hypophysectomized animals and the specific activity relative either to that of the inorganic phosphate of the plasma or to that of the inorganic phosphate of the adrenal itself were decreased 16 hours after the administration of inorganic phosphate labeled with P^{32}. These decreases were largely eliminated by a single injection of ACTH 4 hours before the administration of the P^{32}, i.e. 20 hours before death.

It is concluded that there are at least two changes in the phosphorus metabolism of the adrenal gland of hypophysectomized rats: (a) a slowing in the passage of inorganic phosphate from the extracellular to the intra-

cellular portion of the gland and (b) a slowing in the formation of lipid phosphorus from intracellular inorganic phosphate. It is suggested that both these effects could arise from a decrease in the over-all energy metabolism of the cells of the adrenal glands of hypophysectomized animals.

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NOTES AND COMMENTS

THE ROLE OF PROGESTERONE IN THE PRODUCTION OF VAGINAL CHANGES IN OVARIECTOMIZED FEMALE RATS[1]

Since the work of Allen and Doisy (1923, 1924), cornification of the vaginal epithelium in the ovariectomized mammal has generally been considered to be due to the action of the estrogenic hormones when given in adequate amounts (Robson and Wiesner, 1931; Pincus and Thiman, 1950). When inadequate doses are given, mucification is known to occur (Robson and Wiesner, 1931; Meyer and Allen, 1932). The role of progesterone in the production of vaginal cornification and cyclic changes is less well understood. Hisaw, Meyer and Weichert (1939), among many others, state that administration of progesterone with a cornifying dose of estrogen results in mucification of the vaginal epithelium. Further, the investigations of Hartman and Speert (1941), Jones and Astwood (1942), Everett (1944) and Ford and Young (1951) suggest that progesterone may have a role in vaginal cornification and cyclic changes.

In view of the frequent use made of the rat as an assay animal for the detection of estrogens and of the conflicting views as to the role of progesterone in vaginal cornification, it was thought pertinent to re-evaluate how progesterone, in small doses, might influence this estrogen-induced vaginal cornification in the rat. If it has the same stimulatory effect which it has been shown to have in ovariectomized, estrogen-primed guinea pigs (Ford and Young, 1951), it would indicate that progesterone may have a role in the vaginal cornification of the rat.

METHODS AND RESULTS

Before attempting to determine if progesterone exerts a stimulating effect on vaginal cornification, it was necessary to determine the dosage of estrogen which was just below the threshold required to induce cornification in 50% of the animals treated.

This estrogen dose was determined in ovariectomized Wistar rats which were randomly selected from our colony. The initial amount given was 600 I.U. α-estradiol benzoate. The hormone was dissolved in corn oil and given in 3 divided doses at 24-hour intervals, each dose being larger than the one preceding it. The vaginal contents were aspirated in saline with a bulb pipette, air dried on slides and stained with the Shorr trichrome stain (1941). The animals were treated in such manner for several experiments, being allowed a 3-week resting period between each series of injections. The total dose levels were gradually reduced to 35 I.U. with which 34% of the animals responded, whereas, 90% reacted with 40 I.U.

Once the minimal dose of estrogen was found, it was supplemented by varying doses of progesterone[2] (0.1 to 0.8 I.U.), the latter hormone being given 24 hours after the last estrogen injection. A dose of 0.6 I.U. of progesterone increased the percentage of animals responding from 34% (of 70 animals) to 60% (of 10 animals). This increased cornification is not statistically significant with this small number of animals ($P = .20$ to $.30$ by Chi-square). Further, this increase can not be ascribed to a specific action of progesterone, since this compound was completely ineffective in causing cornification when 0.6 I.U. was given alone in oil.

With a dose of 0.8 I.U. of progesterone, vaginal cornification occurred in only 20% of the animals, the remaining 80% showing variable degrees of mucification. This change was also statistically insignificant due to the small number of animals treated. No dose below 0.8 I.U. decreased the percentage of animals developing vaginal cornification below that observed for the controls. The usual response was a slight, but insignificant increase in the percentage of animals developing cornified vaginal smears.

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[2] Progesterone was supplied by the Schering Corporation, Bloomfield, New Jersey.

Further experiments were performed using the same method of treatment in an attempt to obtain sufficient data for statistical analysis of the role of progesterone in the vaginal cornification of the rat. A different group of females was treated in these studies and found to be refractive to 35 I.U. of estradiol. However, they responded to 50 I.U., at which level 24 of 50 animals developed vaginal cornification. When 0.6 I.U. progesterone (the most effective dose) was given with this amount of estrogen, 30 of 50 animals reacted. This increase in the number of animals cornifying, like that observed previously, was found to be insignificant by the method of Chi-square ($P = .30$ to $.50$).

From these studies it was concluded that no significant increase in cornification occurs as a result of supplementing estrogen treatments of ovariectomized rats with progesterone. Thus, the rat, unlike the guinea pig, appears to have little or no dependence on progesterone for the cyclic changes of the vaginal epithelium.

SUMMARY AND CONCLUSIONS

The results show that progesterone has no consistent or significant stimulating effect on vaginal cornification in the ovariectomized rat when given in conjunction with estrogen. This is in contrast to the important role of this hormone in the vaginal changes of the guinea pig (Ford and Young, 1951). This may in part be due to the differences in the normal luteal activity between the two animals.

It should be emphasized, however, that while cornification was not markedly enhanced by the treatments with progesterone, it was not inhibited until as much as 0.8 of an I.U. was given, which dose caused mucification.

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THE EFFECT OF CORTISONE AND HYDROCORTISONE ON THE TRYPTOPHAN PEROXIDASE-OXIDASE ACTIVITY OF RAT LIVER

In the course of studies on the tryptophan peroxidase-oxidase system of rat liver, we observed that the increase in enzyme activity produced by total-body x-irradiation did not occur after adrenalectomy, and that of a number of substances tested only cortisone and hydrocortisone were capable of eliciting an increase in activity in adrenalectomized rats (Thomson and Mikuta, 1954). We have thought it advisable to present in some detail our incidental observations on the latter point, in view of the possibility that the dose-dependent response of this enzyme system to these steroids may provide a basis for a simple assay method in which for practical purposes a single chemical reaction is involved.

EXPERIMENTAL METHODS AND RESULTS

Two months old Sprague-Dawley or Holtzman female rats, adrenalectomized 5 to 7 days previously and maintained on 1% NaCl, were injected intramuscularly with saline suspensions of cortisone acetate or hydrocortisone acetate. Six hours after injection the rats were decapitated and the livers excised and assayed for enzyme activity by the method of Knox (1951). If the livers were promptly frozen with dry ice and stored at −5° C., no loss in activity occurred for at least 48 hours. The results are shown in Figure 1. It appears that in this

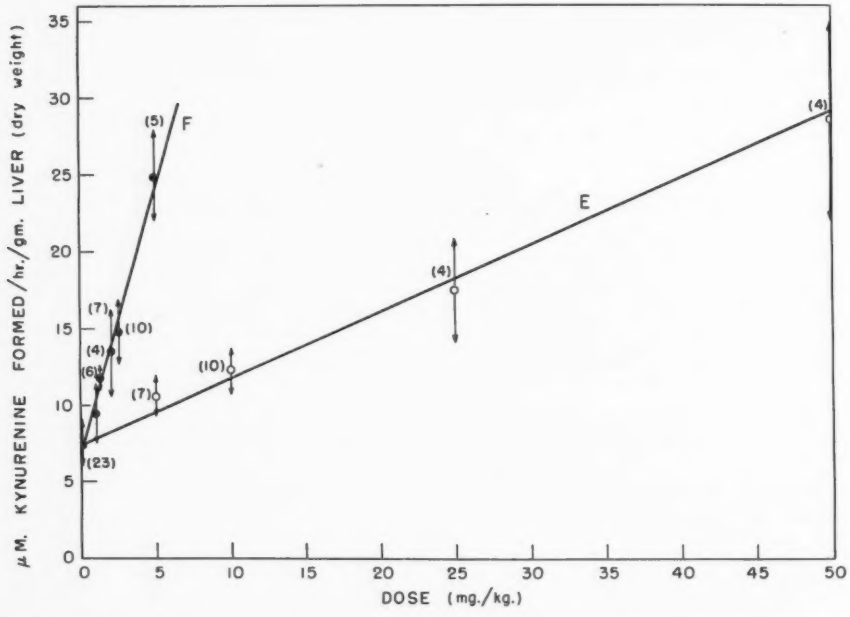

FIG. 1. Effect of cortisone acetate ("E") and hydrocortisone acetate (F) on the activity of adrenalectomized rat liver tryptophan peroxidase-oxidase. Numbers in parentheses indicate the numbers of animals used; vertical arrows represent standard deviations.

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system cortisone is about 1/7 as effective as hydrocortisone. Desoxycorticosterone and tetrahydrocortisone had no effect at doses up to 50 mg./kg. in adrenalectomized rats, and did not significantly modify the response to cortisone or hydrocortisone when administered simultaneously. We have no reliable data on the effects of cortical extracts.

DISCUSSION

No particular care was exercised to ensure uniformity among the rats in regard to age, weight, environment, nutritional state, or even strain (although the two strains used are closely related). If precautions were taken to provide a more nearly homogeneous population, the reliability of the tryptophan peroxidase-oxidase system as an assay method would probably be greatly enhanced. We have noted that hypophysectomized rats seem to be more responsive than adrenalectomized rats to hydrocortisone (Thomson and Mikuta, 1954), but we have no data on the effect of cortisone after hypophysectomy.

SUMMARY

Data are presented to show that the response of the tryptophan peroxidase-oxidase system of rat liver may serve as an assay method for cortical hormones, based on their influence on a discrete biochemical mechanism.

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CERTAIN BIOLOGICAL ACTIVITIES OF CRYSTALLINE ELECTROCORTIN

INTRODUCTION

R. I. Dorfman (1953) mentioned the mouse eosinophil test (Speirs and Meyer, 1951) and the Na^{24}/K^{42} test (Simpson and Tait, 1952) as being sensitive bioassay methods for adrenal hormones, representative of the "carbohydrate" and "mineral" groups respectively. It was, therefore, decided to assay crystalline electrocortin in these two tests in order to obtain the greatest information from the limited amounts of this pure compound so far available. The results are recorded in this paper.

MATERIAL

The crystalline electrocortin used for the Na^{24}/K^{42} test was supplied by Professor Reichstein and prepared by the method already published (Simpson, Tait, Wettstein, Neher, v. Euw and Reichstein, 1953). The crystalline compound used in the eosinophil test was prepared by the English authors of this paper by fractionation of 240 kg. of adrenal glands by methods already described (Simpson and Tait, 1953) followed by final crystallisation from aqueous methanol. These crystals were identical with those supplied by Professor Reichstein as established by investigation of U.V. and infra-red absorption spectra of free compound and acetyl derivatives, of running properties in various Bush

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and Zaffaroni paper chromatographic systems and by comparison of the biological potencies of the free compounds in the Na^{24}/K^{42} test. For reasons previously outlined (Simpson, Tait, Wettstein, Neher, v. Euw, and Reichstein, 1953) these crystals were probably the monohydrate of electrocortin. Their negative reaction with Porter-Silber reagent showed the absence ($<0.5\%$) in this preparation of such steroids as hydrocortisone or cortisone.

METHODS AND RESULTS

Assay in the Mouse Eosinophil Test

The electrocortin was first taken up in absolute ethanol and added to 1% Tween 80 to make a 20% aqueous ethanol solution. This was injected into epinephrine pretreated adrenalectomized BBF_1 mice (Speirs and Meyer, 1951) and eosinophil counts taken at a three hour interval (Speirs, 1952a, 1952b). A standard solution of cortisone acetate was made up in the same way. The results obtained are shown in Table 1A. Because a great deal of variability was obtained, the electrocortin was then taken up in 20% ethanol without the Tween

TABLE 1. EOSINOPHIL ASSAY OF ELECTROCORTIN AND CORTISONE ACETATE IN EPINEPHRINE PRETREATED ADRENALECTOMIZED BBF_1 MICE

	Material	Dose	Volume	No. of animals	Per cent decrease
A	Electrocortin in 1% Tween 80 20% Ethanol	2 μg.	0.05 ml.	3	0
		4 μg.	0.1	3	−21
		10 μg.	0.25	3	−35
B	Electrocortin in 20% Ethanol	10 μg.	0.4 ml.	6	−43 ± 3.2
A	Cortisone Acetate in 1% Tween 80 20% Ethanol	2 μg.	0.05 ml.	8	−4
		4 μg.	0.1	18	−37
		10 μg.	0.25	6	−53
B	Cortisone Acetate in 20% Ethanol	2.5 μg.	0.4 ml.	6	−46 ± 6.1
		5.0	0.4	6	−66 ± 1.4

80 and the dilution adjusted so that a constant volume of 0.4 ml. was injected. The data are shown in Table 1B.

It may be noted that the electrocortin possesses eosinopenic activity approximately one quarter of that shown by cortisone acetate. Although the quantity of available material was relatively small, nevertheless this is believed to be a significant qualitative test. It is hoped that a more quantitative test can be run in the near future.

A small amount of remaining electrocortin (3.6 μg.) was then injected into three protein pretreated normal mice to determine if its eosinopenic activity was a direct one. This procedure is described by Panzenhagen and Speirs (1953). It was noted that the blood eosinophils disappeared more rapidly than did the peritoneal eosinophils, indicating that the action of electrocortin upon eosinophils is probably not direct.

Activity in the Na^{24}/K^{42} test

The compound was injected in 0.1 ml. of 10% aqueous ethanol per rat and assayed at various levels in four and six point assays against desoxycorticosterone. The procedure was otherwise as previously described (Simpson and Tait, 1952) for the assay of other steroids against desoxycorticosterone acetate. Details of the injected doses and the results are shown in Table 2. In all quoted assays, statistical analysis indicated that a valid potency ratio could be calculated. The average value for six such balanced assays gave electrocortin to be 120 times as active as desoxycorticosterone.

DISCUSSION

The activity of electrocortin compared with that of desoxycorticosterone in the Na^{24}/K^{42} assay is of the same order of magnitude as that reported by other workers in similar bioassay methods such as the maintenance of blood urea in the adrenalectomized dog and retention of sodium in the Kagawa test (Simpson, Tait, Wettstein, Neher, v. Euw and Reichstein, 1953), the effect on the urinary inert sodium/potassium ratio in adrenalectomized rats (Mattox, Mason, Albert and Code, 1953) and a growth (modified Grollman) assay (Knauff, Nielson, and Haines, 1953). Nevertheless, it has already been reported (Desaulles, Tripod and Schuler, 1953) that electrocortin, compared with desoxycorticosterone, has a qualitatively different action on water metabolism in a modification of the Kagawa test.

TABLE 2. RESULTS OF SIX BALANCED ASSAYS IN Na^{24}/K^{42} TEST-GRADED DOSES OF DESOXYCORTICOSTERONE AND ELECTROCORTIN

Doses of DOC in μg.	Doses of electrocortin in μg.	Potency ratio μg. DOC = 1μg. electrocortin	Fiducial limits (P = 0.05) in μg. DOC = 1 μg. electrocortin	Index of precision $\frac{s}{b} = \frac{\text{variance}}{\text{slope}}$
1.5; 3.0	0.015; 0.030	107	90–133	0.21
1; 2; 4	0.01; 0.02; 0.04	102	61–172	0.35
1.25; 2.50; 5.00	0.0125; 0.025; 0.050	108	69–174	0.32
1.25; 2.50; 5.00	0.025; 0.025; 0.050	145	83–319	0.41
2; 4	0.01; 0.02; 0.04	117	81–162	0.21
1; 2; 4	0.01; 0.02; 0.04	146	90–301	0.42
Mean		121 ± 8 (S.E.)[1]		0.32 ± 0.03[7] (S.E.)[1]

Investigations of the activity of electrocortin in the "carbohydrate" group of assays has so far not been reported except by one group (Knauff, Nielson and Haines) who recorded a negative result in the liver glycogen and eosinophil assays using a preparation of electrocortin which contained certain impurities including cortisone.

The reasons for the discrepancy with the results reported here are not clear. Since these experiments were started, A. Gordon and R. Gaunt (1954) have also assayed electrocortin and found it to be active in the eosinophil test. These results indicate that the action of electrocortin on carbohydrate metabolism should be further investigated.

SUMMARY

Crystalline electrocortin has been bioassayed in the Na^{24}/K^{42} assay method and in the mouse eosinophil test. Electrocortin is 120 times as active as desoxycorticosterone in its effect on the urinary Na^{24}/K^{42} ratio of adrenalectomized rats. It is about one quarter as active as cortisone acetate in its effect on the depression of circulating eosinophils in the adrenalectomized mouse. The results are compared with other published activities of this hormone.

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BIOLOGICAL STUDIES WITH ELECTROCORTIN (ALDOSTERONE)[1]

Since the pioneer work on the isolation of adrenal cortical steroids, it has been recognized that there probably existed one or more unidentified adrenal substances of exceptionally high biological activity in the "amorphous fraction" of adrenal extracts. More recently Simpson and Tait (1953), and Grundy, *et al.* (1952) detected in adrenal extracts, adrenal perfusates and adrenal vein blood a substance much more potent than any known adrenal steroid in affecting electrolyte excretion. They designated this unidentified material *electrocortin.* A substance with similar properties has been found in human urine by Luetscher and Johnson (1954) and others and in the blood of dogs by Farrell and Richards (1953).

In September of 1953, the first announcement was made of the crystallization of electrocortin from adrenal extracts by Simpson, Tait, Wettstein, Neher, von Euw and Reichstein (1953). Using the same source materials, Mattox, Mason and Albert (1953) reported in October the crystallization of minute quantities of either the same or some similar substance. These chemical accomplishments were made both difficult and notable by the fact that electrocortin is present in any known natural source only in trifling amounts.

The first work on crystalline electrocortin demonstrated its high potency in electrolyte excretion (Desaulles, Tripod and Schuler, 1953) and life-maintenance tests (Gross, 1953; Gross and Gysel, 1954). It is of particular interest, however, in establishing the physiological significance of electrocortin, to determine whether it is only a more potent desoxycorticosterone or whether it shares in addition the properties of other adrenal steroids.

A sample of less than a milligram of electrocortin was made available to us, through the courtesy of Dr. A. Wettstein and associates of Ciba, Ltd., Basle. We undertook to compare it with cortisone in the Selye-Schenker cold stress test and in the eosinophil depletion test. The choice of tests was dictated, first, by the fact that only small amounts of material would be required, and, secondly, by the fact that they were tests in which

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[1] Since this paper was submitted the chemical structure of electrocortin has been established (Simpson, S. A., J. F. Tait, A. Wettstein, R. Neher, J. v. Euw, O. Schindler and T. Reichstein: *Experientia* **10:** 132, 1954) and the substance named *aldosterone.* The latter term should supplant *electrocortin* in references to the crystalline compound.

the 11-17-oxygenated corticoids were presumed to be more effective than desoxycorticosterone. In the cold test it was possible only to use one dose level; in the eosinophil test two dose levels were studied. In all cases electrocortin was put in solution in purified sesame oil and used within 48 hours.

I. Cold Stress Tests. Most published accounts of the cold stress test (Selye and Schenker, 1938; Dorfman, *et al.*, 1946; Vogt, 1943, 1951) concerned use of aqueous cortical extracts or crystalline materials in aqueous solution. In such cases the test material was injected at intervals throughout the period of exposure to cold. We desired to use electrocortin and the free alcohols of cortisone and desoxycorticosterone in oil solution, the slow absorption of which required some modification of the method. This was accomplished by injecting the steroids one hour before exposure to cold, the total dose being divided between the thigh muscles of each leg. 20 μg. doses of each steroid were used. The materials were given at a concentration of 200 μg. per ml. with 15% benzyl benzoate being added to the cortisone solution to achieve solubility. It was established in separate tests that the benzyl benzoate was without effect.

All animals were 22–24 days old and adrenalectomized in the afternoon of the day before the test. Littermates were matched into test groups as evenly as possible on the basis of weight and sex. Since only 30 animals could be studied at one time, electrocortin generally was compared simultaneously only with cortisone and the oil vehicle. The effects of desoxycorticosterone were observed in separate tests, except in Exp. III. In experiments I and IV, the animals were kept at a temperature of 2–5° C. Our rat colony is accustomed to a closely controlled environmental temperature of 23.8° C. and exposure to these low temperatures resulted in such quick deaths (survival of 3.8 hours in control animals) that question could be raised as to the adequacy of time for absorption

TABLE 1. EFFECT OF 20 μg. DOSES OF ELECTROCORTIN AND OTHER ADRENAL STEROIDS ON SURVIVAL OF ADRENALECTOMIZED RATS EXPOSED TO COLD

Exp. No.	No. rats	Treatment	Mean survival Hours ± S.E.[1]	Probability (vs controls)	Survival % controls
I[2]	9	Controls—oil	4.00 ± 0.22	—	—
	9	Cortisone	4.58 ± 0.35	>0.1	+14.5
	9	Electrocortin	4.83 ± 0.33	<0.05	+20.7
II	10	Controls—oil	5.40 ± 0.29	—	—
	10	Cortisone	7.30 ± 0.33	<0.001	+35.2
	9	Electrocortin	7.83 ± 0.52	<0.001	+45.0
III	8	Controls—oil	5.56 ± 0.29	—	—
	8	Cortisone	5.69 ± 0.23	>0.7	+ 2.3
	7	Electrocortin	6.43 ± 0.36	0.08	+15.6
	8	Desoxycorticosterone	5.62 ± 0.54	>0.9	+ 1.1
IV[2]	9	Controls	3.59 ± 0.32	—	—
	9	Desoxycorticosterone	4.00 ± 0.18	>0.1	+11.4
V	10	Controls	4.50 ± 0.28	—	—
	10	Cortisone	6.10 ± 0.47	<0.01	+35.5
	10	Desoxycorticosterone	5.05 ± 0.27	0.2	+12.2
VI	10	Controls	5.05 ± 0.23	—	—
	10	Cortisone	6.31 ± 0.33	<0.01	+25.0
	10	Desoxycorticosterone	5.90 ± 0.24	<0.02	+16.8

[1] S.E. $= \sqrt{\Sigma^2/n(n-1)}$.
[2] Exposed to 2–5° C. temperature; other groups, 6–10° C.

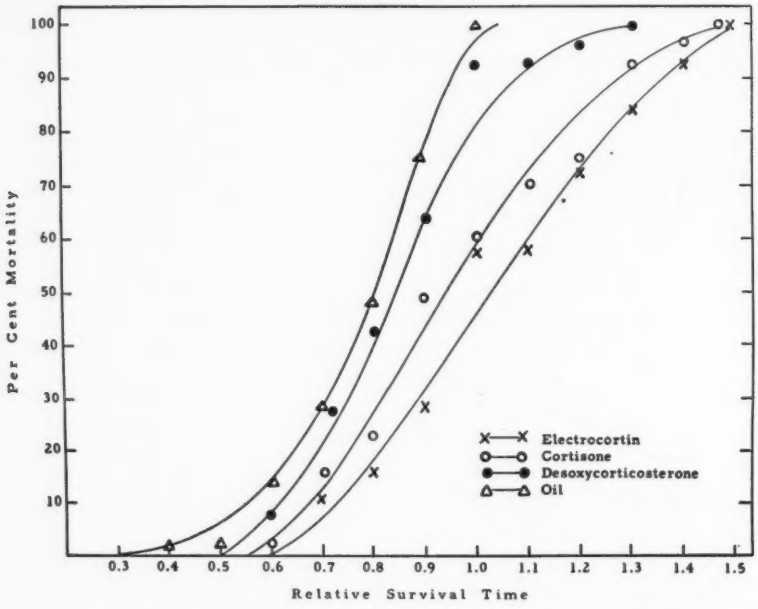

FIG. 1. Percent mortality curves in steroid-treated adrenalectomized rats exposed to cold. Data are pooled from the 5 experiments listed in Table 1. Relative survival times represent values calculated from a common reference point; time of mortality of 100% of oil-injected controls was arbitrarily set at one.

of test materials. Although electrocortin extended life under these conditions, subsequent tests were made at 6–10° C., at which temperature untreated animals lived for an average of 5.1 hours.

Results are shown in Table 1 and Figure 1. In three tests 20 μg. doses of electrocortin extended life longer than in the controls by amounts varying from 16 to 45%. In experiments I and II these differences were significant. In Exp. III, the effect of electrocortin was less certain, although for reasons not known cortisone itself was completely inactive in that experiment. The capricious nature of the cold test and its day-to-day variation have been recognized by others (Dorfman, *et al.*, 1946; Vogt, 1953).

Cortisone was probably active in 4 of 5 tests and certainly so in 3 of 5 tests. In every instance in which they were used simultaneously, however, electrocortin extended life longer than equal doses of cortisone, but the significance of such differences was not established. Desoxycorticosterone may have been mildly beneficial, but actually increased survival significantly only in 1 of 3 tests. This steroid had been reported previously to be active in the cold test only if given 12 hours before exposure to cold (Zarrow, 1942). Electrocortin could thus be differentiated from desoxycorticosterone by the speed as well as by the magnitude of its protective action.

II. Eosinophil Depletion Tests. The techniques recommended by Speirs and Meyer (1949, 1951) have been followed in assessing the eosinopenic activity of electrocortin. BBF_1 male hybrid mice, secured from the Jackson Memorial Laboratory, Bar Harbor,

Maine, were adrenalectomized and injected immediately with 2.5 mg. desoxycorticosterone trimethylacetate. They were employed for the eosinophil test no earlier than 5 days following the operation. Eosinophil numbers were estimated from freely flowing tail blood samples diluted with Speirs' fluid and the cells were counted in 4 chambers of the Speirs eosinophil counting slide.

In each case, an initial count was made, following which a subcutaneous injection of 20 μg. epinephrine HCL in 0.1 ml. saline was administered in the dorsal anterior region. A second count was made 4 hours later. Animals which displayed a drop in eosinophil numbers at this time were discarded.

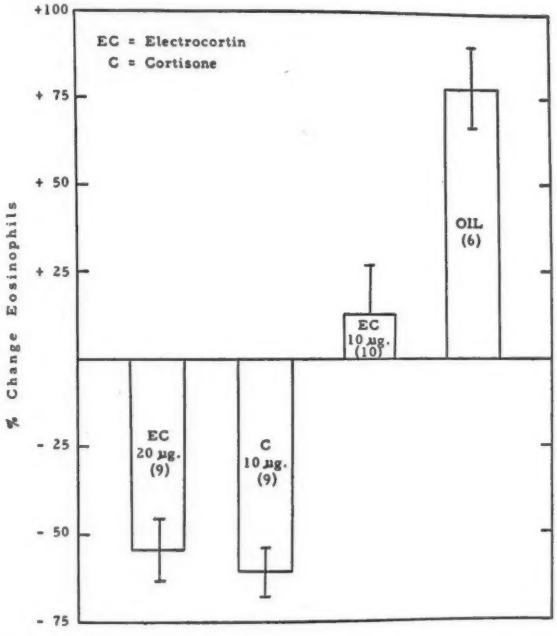

FIG. 2. Showing the changes in eosinophil levels caused by electrocortin and cortisone in epinephrine-treated adrenalectomized mice. The number of animals used in each group is indicated in parentheses.

Electrocortin, as a single dose of 10 μg. or 20 μg., was administered subcutaneously in the dorsal posterior region. A third count was obtained 3 hours later. The percentage change in eosinophil numbers, compared to the second count, was calculated. Animals injected with 10 μg. of cortisone or with the oil vehicle served as controls.

It is apparent from Figure 2 that 20 μg. of electrocortin possesses approximately the same eosinopenic activity as 10 μg. of cortisone. Although electrocortin, in the 10 μg. dosage depressed the eosinophil counts in only 2 of the 10 animals tested, it was effective in preventing the rise in counts seen consistently in the vehicle-injected controls. Speirs (personal communication) has also found eosinopenic activity with electrocortin.

DISCUSSION

The cold stress test is one in which all corticoids previously examined, including the "amorphous fraction" (Kendall, 1940), exert demonstrable activity, although desoxycorticosterone is weak and must be given some hours before exposure to cold (Zarrow, 1942). The eosinophil depletion test, used with the precautions devised by Speirs, can be employed to differentiate between the 11-oxy and 11-desoxycorticoids at low dosage levels. It does not necessarily differentiate between them at high dose levels, since 100 μg. of DCA may cause eosinopenia (Speirs and Meyer 1951).

The results reported here, together with those of others, suggest that electrocortin possesses in varying degrees the biological properties of both the 11-17-oxygenated and 11-desoxy types of adrenal steroids. Thus, it is some 30 times as active as desoxycorticosterone in causing sodium retention, but only 5 times as active in causing potassium excretion (Desaulles, *et al.*, 1953). It is approximately 25–30 times as potent as desoxycorticosterone in maintaining adrenalectomized dogs (Gross, 1953, 1954; Swingle, *et al.*, 1954). Very recently, it has been shown to have about one-third the activity of cortisone and 30 times the activity of desoxycorticosterone in causing liver glycogen deposition (Schuler, Desaulles and Meier, 1954). In the cold stress test used here electrocortin was if anything more active than cortisone and in causing eosinopenia its activity approximated half that of cortisone.

SUMMARY

Electrocortin, the newly crystallized hormone from the adrenal cortex, is known to be much more potent than desoxycorticosterone in affecting electrolyte excretion. It was found, in addition, to have an activity equal to or greater than cortisone in the cold stress test and to be active, although weaker than cortisone, in the eosinophil depletion test. This together with other available evidence indicates that electrocortin has a combination of biological properties, the ratios of which distinguish it from other known cortical steroids.

ADDENDUM

Since the above was written, the authors have found that aldosterone has approximately one-third the activity of cortisone in suppressing a stress-induced release of ACTH in rats.

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CIBA AWARD OF 1954

The Endocrine Society announces the presentation of the Ciba Award of 1954 to Dr. Isador Nathan Rosenberg of Boston.

This award carries a stipend of $1,800 with an additional $700 should the recipient, within twenty-four months of the date of the award, choose to use it toward further study in a laboratory other than that in which he is at present working. This award, which was established in 1942, is given annually to an investigator not more than 35 years of age in recognition of meritorious accomplishments in the field of clinical or preclinical endocrinology.

Dr. Rosenberg has been engaged in research, clinical work, and teaching at the New England Center Hospital and Tufts College Medical School in Boston. Most of his more recent independent investigations in his current post have been concerned with the thyroid and pituitary glands. He developed new chromatographic methods for the separation and isolation of the iodine-containing compounds of the thyroid gland and for this work he was awarded the Van Meter Prize of the American Goiter Association three years ago. Chromatographic methods were also applied to the problem of the nature of the thyroid hormone in Graves' Disease. Dr. Rosenberg showed that this is thyroxine, thus dispelling the older idea of some mysterious poisonous secretion from the thyroid gland in thyrotoxicosis. The mechanism of action of antithyroid drugs was clarified by the simultaneous and independent discovery by Dr. Rosenberg and by Arnott and Doniach that all classes of antithyroid compounds interfere with peroxidase action, strongly supporting the theory that the first step in thyroid hormone synthesis is an oxidation brought about by a peroxidase-like enzyme in the gland.

Dr. Rosenberg furthered the development of more effective forms of corticotropin and of longer acting forms for clinical use and was the first to show that oxycellulose is a good absorbing medium for the hormone, and that purified corticotropic preparations exerted potent metabolic effects.

Dr. Rosenberg was one of the first to apply the technic of electrophoresis on paper to the study of lipoproteins and was able to show that the incidence of atherosclerosis bore no relationship to the pattern of lipoproteins in the serum. A similar technic was profitably applied to the analysis of the components of pituitary hormone preparations.

THE 1955 ANNUAL MEETING

The Thirty-seventh Annual Meeting of The Endocrine Society will be held in the Chalfonte-Haddon Hall Hotel, Atlantic City, New Jersey, on Thursday, Friday, and Saturday, June 2–3–4, 1955.

Dr. Matthew Molitch, 705 Pacific Avenue, Atlantic City, New Jersey, is in charge of the local arrangements for the meeting.

All Scientific Sessions will be held in the Chalfonte-Haddon Hall Hotel. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The annual dinner is scheduled for Friday, June 3, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Make reservations directly with the Chalfonte-Haddon Hall, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

Those wishing to present papers, which will be limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. E. B. Astwood, 30 Bennet Street, Boston 11, Massachusetts, not later than February 1, 1955. It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program and Journals of the Society. The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes nor acknowledgments to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be given after his name.
   Line 3. Institution of origin and city in which institution is located.
3. The body of the abstract, typed double-space, should follow the heading. The original copy should be on bond paper. There should be three copies.

4. Abstracts should be letter-perfect, since there will be no opportunity for proof reading by authors.
5. Abstracts not conforming to the above will be returned to the authors.

Urology Award

The American Urological Association offers an annual award of $1000 (first prize of $500, second prize $300 and third prize $200) for essays on the result of some clinical or laboratory research in Urology. Competition shall be limited to urologists who have been graduated not more than ten years, and to men in training to become urologists.

The first prize essay will appear on the program of the forthcoming meeting of the American Urological Association, to be held at the Biltmore Hotel, Los Angeles, California, May 16–19, 1955.

For full particulars write the Executive Secretary, William P. Didusch, 1120 North Charles Street, Baltimore, Maryland. Essays must be in his hands before January 1, 1955."

Applications for Grants in Cancer Research

Acting for the American Cancer Society, the Committee on Growth of the National Research Council is accepting applications for grants-in-aid for cancer research in the United States. Applications received before October 1 will be considered during the winter and grants recommended at that time become effective on July 1, 1955. Investigators now receiving support will be notified individually regarding their application for renewal.

The Committee feels that an understanding of cancer depends upon a deeper insight into the nature of the growth process, normal and malignant. Therefore, the scope of the research program is broad and includes, in addition to clinical investigations on cancer, fundamental studies in the fields of cellular physiology, morphogenesis, genetics, virology, biochemistry, metabolism, nutrition, cytochemistry, physics, radiobiology, chemotherapy, endocrinology and environmental cancer.

Application blanks and additional information may be obtained from the Executive Secretary, Committee on Growth, National Research Council, 2101 Constitution Avenue, N.W., Washington 25, D.C.